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Chandler Fulton, et al.

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## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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1. ☐ Fee Transmittal Form  
(Submit an original, and a duplicate for fee processing)  
Specification [Total Pages 50 ]
2. ☒ (preferred arrangement set forth below)  
- Descriptive title of the Invention  
- Cross References to Related Applications  
- Statement Regarding Fed sponsored R & D  
- Reference to Microfiche Appendix  
- Background of the Invention  
- Brief Summary of the Invention  
- Brief Description of the Drawings (if filed)  
- Detailed Description  
- Claim(s)  
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 USC 113) [Total Sheets 9 ]

4. ☐ Oath or Declaration [Total Pages ]

- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from a prior application (37 CFR 1.63(d)  
(for continuation/divisional with Box 17 completed)  
[Note Box 5 below]

i. ☐ DELETION OF INVENTOR(S)

Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

5. ☐ Incorporation By Reference (useable if Box 4b is checked)  
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

## ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)
10. ☐ English Translation Document (if applicable)  
Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations  
and Form 892  
Per Rule 97(d) copies of those documents are not provided.
11. ☐
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired
15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. \* ☐

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No:

## 18. CORRESPONDENCE ADDRESS

Wesley B. Ames  
Registration No. 40,893Brobeck, Phleger & Harrison LLP  
12390 El Camino Real  
San Diego, CA 92130  
Telephone: (858) 720-2500  
Facsimile: (858) 720-2555

- ☒ If a paper is untimely filed in the above-referenced application by applicant or his/her representative, the Commissioner is hereby petitioned under 37 C.F.R. § 1.136(a) for the minimum extension of time required to make said paper timely. In the event a petition for extension of time is made under the provisions of this paragraph, the Commissioner is hereby requested to charge any fee required under 37 C.F.R. § 1.17(a)-(d) to **Deposit Account No. 50-1273**. However, the Commissioner is **NOT** authorized to charge the cost of the issue fee to the Deposit Account.

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Dated:

9/29/08

Respectfully submitted,

By:

  
Wesley B. Ames

Registration No. 40,893

Brobeck, Phleger & Harrison LLP  
12390 El Camino Real  
San Diego, CA 92130  
Telephone: (858) 720-2500  
Facsimile: (858) 720-2555

## **DESCRIPTION**

### **THIAMINASES AND THIAMINASE GENES FOR USE IN APOPTOTIC THERAPIES**

5

This application is related to Fulton & Lai, U.S. Patent Application 09/113,596, filed July 10, 1998, which claims the benefit of Fulton et al., U.S. Provisional Application 60/052,377, filed July 11, 1997, and of Fulton et al., U.S. Provisional Application 60/087,526, filed June 1, 1998, all entitled METHOD OF  
10 INDUCING APOPTOSIS BY REDUCING THE LEVEL OF THIAMIN, all of which are incorporated by reference in their entireties, including drawings.

### **Background of the Invention**

#### **1. Field of the Invention**

15 This invention is related to the field of treatment of cancer, other neoplastic disorders, and other conditions in vertebrates in which killing a specific group of cells is useful., and in particular to the use of thiamin-cleaving enzymes, thiaminases, and their expressed thiaminase genes, as a means to induce apoptotic death of targeted cells, such as cancer cells, by reducing the level of thiamin  
20 (vitamin B1) in these cells.

#### **2. Background Information**

The information provided herein is intended to aid the understanding of the reader, and is not an admission that any of the information or references is prior art  
25 to the present invention.

Most if not all cells of metazoan animals carry the machinery to commit suicide in a regular manner in response to suitable stimulus. This process is called programmed cell death, cell suicide, or apoptosis. Apoptosis is being extensively studied in mammals and other vertebrates, as well as in the worm *Caenorhabditis*  
30 *elegans* and the fly *Drosophila melanogaster* (reviews: Ellis et al., 1991; Steller,

1995). In vertebrate cells the process of apoptosis, which was previously termed “shrinkage necrosis,” involves a regular sequence of events, including membrane blebbing, cell shrinkage, pycnosis of nuclei with margination of chromatin, and usually cleavage of DNA into nucleosome-sized fragments (Wyllie et al., 1980).

- 5 Apoptosis is an essential part of embryonic development and of the maintenance of an adult animal. In mammals, for example, during development apoptosis plays a major role in the development of the nervous system (more than 50% of the neural cells that arise during embryogenesis undergo apoptosis), in the elimination of lymphocytes that produce antibodies which recognize self, in  
10 “carving” features such as the digits of the hand, and so forth. Throughout life, orderly apoptosis is used to eliminate damaged or unwanted cells without inducing an inflammatory reaction. Blood cells, cells of the immune system, and cells of most if not all tissues normally are eliminated by the apoptotic mechanism.

- Failures of apoptosis produce or contribute to severe diseases, including  
15 autoimmune diseases and some cancers. It has been argued that one of the major causes of the development and progression of many cancers is a reduction of the occurrence of apoptosis (Wyllie, 1985; Fisher, 1994; Hickman et al., 1994; Martin and Green, 1995; Thompson, 1995).

- A wide variety of signals induce apoptosis in suitable target cells  
20 (Gerschenson and Rothello, 1992; Thompson, 1995). Radiation and many valuable chemotherapeutic agents, such as cisplatin and other platinum compounds, induce apoptosis (e.g., Eastman, 1990; Hickman, 1992; Chu, 1994a). These agents affect many cell types. Specialized cell types are dependent on specific growth factors (e.g., nerve growth factor for certain neuronal cells, interleukin-2 for certain  
25 lymphocytes) and undergo apoptosis if the required factors are unavailable. Other cell types have receptors for specific agents that can induce apoptosis in these cell types (e.g., glucocorticoid for thymocytes, tumor necrosis factor in suitable target cells) (e.g., Rubin et al., 1988).

- The mechanism of apoptosis is just beginning to be understood. Some have  
30 suggested that all cells are poised to die, and that they are kept alive by constant “survival signals” that keep the suicide machinery inactive (Raff, 1992). It is clear that many if not all vertebrate cells contain preformed machinery for apoptosis, since there are many examples of cells that undergo apoptosis even without synthesis of

new proteins (Waring, 1990). There also are cases in which protein synthesis is required (reviewed by Cohen, 1993).

Several elements that appear to be part of the apoptotic machinery have been identified and are receiving much attention. Two that should be mentioned are bcl-2 and its family members and p53. Exactly how these are related to the apoptotic machinery is still being defined.

Expression of oncogene bcl-2 in cells markedly delays or blocks induction of apoptosis by many agents, including some that are valuable in chemotherapy of tumors, such as cisplatin (Reed, 1994; Korsmeyer, 1995; Thompson, 1995). There are a few cases in which induction of apoptosis is unaffected by expression of bcl-2 (e.g., Sentman et al., 1991; Vaux et al., 1992). High-level expression of bcl-2 is common in tumors, including breast carcinomas, small cell lung cancer, androgen-independent prostate cancer, and neuroblastoma (Hickman et al., 1994). In some cases expression of bcl-2 is correlated with a poor prognosis for therapy (Reed, 1994).

Functional tumor suppressor gene product p53 is required for induction of cell death by irradiation and many chemotherapeutic agents (Lowe et al., 1993), as well as by oxygen deficiency at the center of solid tumors (Graeber et al., 1996). On the other hand, the normal development of transgenic animals nullizygous for the p53 gene indicates that p53 is not required for the extensive apoptosis that occurs during development (Donehower et al., 1992). Other cases of p53-independent apoptosis have been described (White, 1993; Zhuang et al., 1995). Many established lines of cells in culture have lost p53 function. In tumors *in vivo*, loss of p53 function is common, and this loss is correlated with tumor aggressiveness and indicates a poor prognosis for treatment by standard protocols of chemotherapy and radiation (Fisher, 1994; Hartmann et al., 1997).

As an example, the roles of p53 loss and bcl-2 expression in the development and progression of colon carcinomas have been described and analyzed (Hickman et al., 1994; Sinicrope et al., 1996).

A previous patent application (Pat1) described methods for inducing apoptosis of a selected group of cells *in vivo* by reducing the level of thiamin in these cells. Methods for inducing apoptosis of cancer cells were included. Compounds and

compositions for use as methods of thiamin depletion and treating disease such as cancer were also described.

### Summary of the Invention

5           The present invention is based on our discovery that apoptotic cell death can be induced in diverse cell types by creating a deficiency in the natural vitamin, thiamin and brings together three disparate areas: *a)* the programmed death or apoptosis of vertebrate cells, *b)* an agent in the unicellular protist *Naegleria* and in certain other organisms that induces delayed apoptosis, even in quiescent cells, and  
10       *c)* thiamin deficiency.

          The invention provides a method for inducing death in selected cells *in vivo* by using localized delivery of thiamin-depleting compounds to reduce the thiamin in these cells below a critical level. This method, localized apoptosis induced by depletion of thiamin (LAIDT), is applicable to therapy of cancer and to elimination  
15       of other targetable cells. Furthermore, the method allows rapid and convenient reversal of the effects of the deficiency at any time such reversal is desired, simply by the administration of replacement thiamin.

          This method allows the selective killing of a group of cells, for example a tumor mass, by localizing the deficiency of thiamin to the desired cell group. Both  
20       the thiamin depletion and the targeting can be accomplished in a variety of different ways, as described in Fulton et al., U.S. Patent Application 09/113,596, filed July 10, 1998, and International application No. PCT/US98/14496, both entitled METHOD OF INDUCING APOPTOSIS BY REDUCING THE LEVEL OF THIAMIN. Typically however, the method involves the delivery of a thiamin-  
25       depleting agent or a nucleic acid sequence encoding a thiamin-depleting agent to the desired cell group. The creation of the thiamin deficiency, which results from the delivery of the thiamin-depleting agent, leads to programmed cell death, or apoptosis. This method is broadly applicable to use with cells of vertebrate organisms, which cannot produce their own thiamin and so rely on exogenously  
30       provided, i.e., dietary, thiamin to provide the cellular requirements. In particular, the method can be utilized *in vivo* in a vertebrate organism, for example a human.

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This invention utilizes a novel paradigm for cancer therapy, in addition to those currently commonly used or tested (e.g., radiation, chemotherapy, immunotherapy, gene therapy, and antiangiogenesis therapy). In this paradigm, selective starvation of cancer cells for a particular required nutrient whose absence induces apoptosis, in this description the essential vitamin, thiamin, leads to death of the cancer cells.

Thus in a first aspect, the invention provides a method for inducing apoptosis of a selected group of vertebrate cells *in vivo* by sufficiently reducing the level of thiamin in cells of the group, by administering a thiaminase or a thiaminase derivative. For example, the cells may be neoplastic cells, e.g., cancer cells.

Preferably the thiaminase is a eukaryotic thiaminase or a Type 2 thiaminase, or derivative thereof. In particular embodiments, the thiaminase is a *Naegleria gruberi* thiaminase or a *Bacillus thiaminolyticus* thiaminase, or a *Clostridium sporogenes* thiaminase, or derivative thereof.

Although emphasis herein is on use of LAIDT for cancer therapy, thiamin deficiency and the methodology herein allows therapeutic elimination of other unwanted cells.

Thiaminases are described in detail in Fulton et al., U.S. Patent Application 09/113,596, and PCT/US98/14496, and are a preferred embodiment for LAIDT along with derivatives of thiaminases. Most organisms do not appear to have any thiaminase activity. There are two types, thiaminase I that cleaves thiamin by a base-substitution reaction and the rarer thiaminase II that cleaves thiamin by direct hydrolysis (reviewed by (Evans, 1975; Fujita, 1954)). None has been found in mammals or birds (e.g., Harris, 1951; Puzach, 1991). Thiaminase I has been found in some but not all shellfish, some fresh-water fish (especially of the carp family), a few plants (especially ferns such as bracken), one protozoan (*Naegleria gruberi*), and scattered bacteria (including *Bacillus thiaminolyticus* and *Clostridium sporogenes*). Prior to this invention, only the thiaminase I of *Bacillus thiaminolyticus* has been cloned ((Abe et al., 1987)) or sequenced ((Costello et al., 1996)) (DNA sequence no. U17168).

In another aspect, the invention provides a pharmaceutical composition containing a nucleic acid molecule encoding a thiaminase or a thiaminase derivative and a pharmaceutically acceptable carrier or excipient.

5 In another aspect, the invention provides an isolated, purified, or enriched nucleic acid molecule encoding a non-*Bacillus thiaminolyticus* thiaminase, or a derivative (including a fragment) of such a thiaminase with thiamin-cleaving and/or thiamin-binding activity. Preferably the thiaminase or derivative is a *Naegleria gruberi* thiaminase or derivative.

10 In preferred embodiments, the nucleic acid molecule encodes a thiaminase or thiaminase derivative homologous to a *Naegleria gruberi* thiaminase.

By "homologous" is meant that a particular sequence from a thiaminase or thiaminase gene had at least 70% sequence identity, as defined by a maximal base match in a computer-generated alignment, at the nucleotide level with the reference  
15 sequence, preferably at least 80%, and more preferably at least 85%, over at least a 50 nucleotide window, preferably over at least a 75 or 100 nucleotide window, more preferably over at least a 200, 300, 400, or 500 nucleotide window, and most preferably over the entire coding sequence or over the entire gene. Likewise, homology can be shown by at least 25% sequence identity at the polypeptide level  
20 as compared to a reference sequence, preferably at least 35%, 40%, 45%, 50%, 55%, 60%, 70%, or even more, or by sequence similarity of at least 45%, preferably at least 50%, 60%, 70%, 80%, or even more.

For nucleotide or amino acid sequence comparisons where a homology is defined by a % sequence identity, the percentage is determined using BLAST  
25 programs (with default parameters (Altschul et al., 1997, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acid Res. 25:3389-3402)). Any of a variety of algorithms known in the art that provide comparable results can also be used, preferably using default parameters. Performance characteristics for three different algorithms in homology searching is  
30 described in Salamov et al., 1999, "Combining sensitive database searches with multiple intermediates to detect distant homologues." *Protein Eng.* 12:95-100. Another exemplary program package is the GCG™ package from the University of Wisconsin.



In preferred embodiment, the thiaminase is from a fern or other pteridophyte, such as the fern bracken (*Pteridium aquilinum*) or the fern nardoo (*Marsilea drummondii*). In still another, the thiaminase is from a fish, preferably of the family Cyprinidae, such as carp.

5 In the case of nucleic acid sequences encoding thiaminase derivatives, it is often advantageous for the encoded amino acid sequence to be shorter than a full length naturally occurring thiaminase. Therefore, in preferred embodiments, the nucleic acid sequence encodes a modified thiaminase or thiaminase derivative containing about 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, or  
10 40% or less or the amino acid sequence of the corresponding natural thiaminase. Thus, for example, the nucleic acid sequence can encode a derivative having about 400 or fewer, 200 or fewer, 100 or fewer, or 50 or fewer amino acids. Similarly, the nucleic acid sequence can encode a polypeptide thiamin-binding compound or derivative.

15 By "isolated" in reference to nucleic acid is meant a polymer of nucleotides conjugated to each other, including DNA or RNA that is isolated from a natural source or that is synthesized. The isolated or synthesized (e.g., cDNA) nucleic acids of the present invention are unique in the sense that they are not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally  
20 occurring sequence has been removed from its normal cellular (*i.e.*, chromosomal) environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide sequence present, but that it is essentially free (about 90 - 95% pure at least) of non-nucleotide material naturally associated with it and thus is meant to  
25 be distinguished from isolated chromosomes.

By the use of the term "enriched" in reference to nucleic acid is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction (2 - 5 fold greater, more preferably >100-fold) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from  
30 which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that "enriched" does not imply that there are no

other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term "significant" here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2 fold, more preferably at least 5- to 10-fold, more preferably at least 100- to 1000-fold, or even more. The term also does not imply that there is no DNA or RNA from other sources. The other source DNA may, for example, comprise DNA from a yeast or bacterial genome, or a cloning vector. This term distinguishes the sequence from naturally occurring enrichment events, such as viral infection, or tumor type growths, in which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant to cover only those situations in which a person has intervened to elevate the proportion of the desired nucleic acid.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, *e.g.*, in terms of mg/ml, more preferably at least 100- or 1000-fold greater). Individual clones isolated from a cDNA or genomic library may be purified to electrophoretic homogeneity. The claimed DNA molecules obtained from these clones can be obtained directly from total DNA or from total RNA. The cDNA clones or genomic are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection of the cells carrying the cDNA library. Thus, the process which includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximately  $10^6$ -fold purification of the native message. Thus, purification of at least three orders of magnitude, and more preferably four or five orders of magnitude is expressly contemplated. The term is also chosen to distinguish clones already in existence which may encode a thiaminase or portion of a thiaminase but which have not been isolated from other clones in a library of

clones. Thus, the term covers clones encoding a thiaminase or portion of a thiaminase which are isolated from other non-thiaminase clones.

A polypeptide thiaminase can be encoded by a full-length nucleic acid sequence or portion of the full-length nucleic acid sequence. In preferred  
5 embodiments the isolated nucleic acid comprises, consists essentially of, or consists of a nucleic acid sequence encoding a naturally-occurring thiaminase, a nucleic acid sequence that hybridizes to such a nucleic acid sequence, or a functional derivative of either. The nucleic acid may be isolated from a natural source by cDNA cloning, use of PCR primers, subtractive hybridization, or other means standard to the art; the  
10 natural source may be any organism which naturally produces a thiaminase, specifically including those described in the Detailed Description below, and the nucleic acid may be synthesized by the triester or other method or by using an automated DNA synthesizer.

The term "hybridize" refers to a method of interacting a nucleic acid  
15 sequence with a DNA or RNA molecule in solution or on a solid support, such as cellulose or nitrocellulose. If a nucleic acid sequence binds to the DNA or RNA molecule with high affinity, it is said to "hybridize" to the DNA or RNA molecule. The strength of the interaction between the probing sequence and its target can be assessed by varying the stringency of the hybridization conditions. Various low or  
20 high stringency hybridization conditions may be used depending upon the specificity and selectivity desired. Stringency is controlled by varying salt or denaturant concentrations. Examples of hybridization conditions are shown in the examples below. Those skilled in the art will recognize how such conditions can be varied to vary specificity and selectivity. Under highly stringent hybridization conditions  
25 only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having one or two mismatches out of 20 contiguous nucleotides.

The invention also features recombinant nucleic acid encoding a thiaminase or thiaminase derivative, preferably in a vector effective to initiate transcription in a  
30 host cell. The vector may be in such a eukaryotic host cell or *in vivo* in cells of an organism. The recombinant nucleic acid can, for example, contain a transcriptional initiation region functional in a cell, a sequence complementary to an RNA sequence encoding a thiamin-depleting agent polypeptide and a transcriptional termination

region functional in a cell. While recombinant nucleic acid encoding an unmodified thiaminase, for example in a eukaryotic expression vector, from *Bacillus thiaminolyticus* can be utilized in the methods of this invention, in certain embodiments the encoded thiaminase agent is different from that enzyme, e.g., a eukaryotic thiaminase and in other embodiments is not a modification or derivative of that thiaminase.

Thus, in a related aspect the invention provides a eukaryotic expression vector which includes a nucleic acid sequence encoding a thiaminase or thiaminase derivative. The expression vector is constructed and adapted for expression in eukaryotic cells, preferably in human cells. Preferably the vector does not include an origin of replication functional in eukaryotic cells. While vectors based on viral sequences can be beneficially used, in preferred embodiments, the vector is a non-viral vector, meaning that the vector does not contain sufficient viral sequences to cause viral replication or capsid formation. In certain embodiments, the encoded thiamin-depleting agent differs from a full-length thiaminase from *Bacillus thiaminolyticus* and in other embodiments is not a modification or derivative of that thiaminase. Other preferred embodiments are as described above for the nucleic acids and nucleic acid delivery methods.

In another related aspect, the invention provides a vector which includes a recombinant nucleic acid sequence which encodes a polypeptide thiaminase or a thiaminase derivative which is different from a *Bacillus thiaminolyticus* thiaminase. In preferred embodiments, the vector is an expression vector which is constructed and adapted for expression in prokaryotic cells, for example, *E. coli*, though a variety of other bacteria can be used. In other embodiments the vector is a eukaryotic expression vector, which is constructed and adapted for expression in eukaryotic cells. Other preferred embodiments are as described for the vectors, nucleic acids and nucleic acid delivery methods above.

In accord with the vectors and methods for delivery of nucleic acid encoding a thiaminase, the invention also provides a eukaryotic cell transfected with a eukaryotic expression vector containing a nucleic acid sequence encoding a thiaminase. Preferably, the cell is a vertebrate cell *in vivo* in a vertebrate organism, such as a bird or a mammal, e.g., a human. The thiaminase can be any peptide or polypeptide compound, such as those described in the above aspects.

In another related aspect, the invention provides a composition for delivery of a nucleic acid sequence encoding a thiaminase or a thiaminase derivative to vertebrate cells *in vivo*. The composition includes a nucleic acid sequence encoding the thiaminase or a thiaminase derivative. The composition preferably also includes  
5 a component associated with a nucleic acid sequence which enhances delivery of the nucleic acid into the cells. In preferred embodiments, the nucleic acid and other components of the composition are as described above in connection with methods involving delivery of a nucleic acid sequence.

Thiaminases and derivatives obtained from natural sources will be useful as  
10 described for the methods of this invention, and for analysis for constructing derivatives and synthetic thiamin-cleaving compounds. Thus, another aspect of the invention features an isolated, enriched, or purified polypeptide thiaminase which has not previously been obtained. In the case of an agent which has been enriched or partially purified, the invention provides a purified agent, so that the agent is  
15 separated from at least 95%, preferably from at least 98%, and still more preferably from at least 99% of the macromolecules from the environment in which the agent is naturally produced. The agent therefore differs from a *Bacillus thiaminolyticus* thiaminase or mutated form of that thiaminase involving substitution or deletion of less than 1%, 5%, or 10% of the amino acid sequence of that thiaminase. In  
20 preferred embodiments, the agent is a thiaminase or thiaminase derivative.

The invention also provides an isolated, purified, or enriched nucleic acid molecule that has a nucleotide sequence at least 90% identical, preferably at least 95%, 97%, 98%, 99%, or 100% to a portion of a *Naegleria gruberi* thiaminase gene or coding sequence at least 15, 17, 20, 25, 30, 35, 40, 50, 75, 100, 200, or even more  
25 nucleotides in length.

In another aspect, the invention provides an isolated, purified, or enriched non-Bacillus thiaminolyticule thiaminase polypeptide, or a derivative thereof having thiamin-cleaving and/or thiamin-binding activity.

30 By "isolated" in reference to a polypeptide is meant a polymer of 6, 12, 18 or more amino acids conjugated to each other, including polypeptides that are isolated from a natural source or that are synthesized. In this invention, the polypeptide will commonly have at least about 50, 100, 200, or 400 amino acids conjugated together.

The isolated polypeptides of the present invention are unique in the sense that they are not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally occurring sequence has been removed from its normal cellular environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only amino acid chain present, but that it is essentially free (about 90 - 95% pure at least) of material naturally associated with it.

By the use of the term "enriched" in reference to a polypeptide it is meant that the specific amino acid sequence constitutes a significantly higher fraction (2- to 5-fold greater) of the total of polypeptide present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other polypeptides present, or by a preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that "enriched" does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other polypeptides of about at least 2-fold, more preferably at least 5 to 10 fold or even more. The term also does not imply that there is no polypeptide from other sources. The other source polypeptide may, for example, comprise amino acid encoded by a yeast or bacterial genome, or a cloning vector. The term is meant to cover only those situations in which a person has intervened to elevate the proportion of the desired polypeptide.

It is also advantageous for some purposes that an amino acid sequence be in purified form. The term "purified" in reference to a polypeptide does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, *e.g.*, in terms of mg/ml). Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

In another aspect, the invention provides a method for identifying a nucleic acid sequence coding for a thiaminase by probing for sequences which hybridize with a nucleic acid probe that contains a sequence which is the same as a sequence from a thiaminase-encoding gene of *Naegleria gruberi* or *Bacillus thiaminolyticus*. Preferably the probe contains a nucleic acid sequence at least 10 nucleotides in length with the same base sequence as the equal length sequence from the specified organism and/or a degenerate sequence that contains alternate codon usage at one or more of the codons. In preferred embodiments, the probe contains a longer sequence corresponding (the same or utilizing alternate codon usage) to the *Naegleria gruberi* or *Bacillus thiaminolyticus* thiaminase sequences or derivatives, for example, at least 12, 13, 15, 17, 20, 25, 30, 40, 50, or even more nucleotides. Preferable a plurality of probes is used that may all correspond to the same *Naegleria gruberi* or *Bacillus thiaminolyticus* sequence or may correspond to more than one such sequence or both. The probe or probes is used to hybridize to a complementary target sequence in nucleic acid from a different organism which is being tested for the presence of a thiaminase-encoding sequence, thereby identifying such a sequence if present. The target sequence need not be perfectly complementary; useful hybridization results can be provided, for example, by hybridization of complementary sequences that have 7 out of 10, 8 out of 10, 9 out of 10, or 10 out of 10 base pairing over the target sequence. The target sequence is generally in a cDNA or genomic DNA clone library, and hybridization identifies a clone insert containing the target sequence. As recognized by those skilled in the art, hybridizing clones can be sequenced and expressed using standard materials and methods to identify a coding sequence and product. If needed, additional libraries or sub-libraries can be constructed by conventional methods to identify larger or preferably full-length coding sequences.

Alternatively, for organisms for which sequence information is available, instead of probing with degenerate probe sets, sequence comparison can be used using any of the publicly available computer-based software for polynucleotide or polypeptide sequence comparisons to identify sequences which have levels of sequence similarity indicative of thiaminase function. Those accustomed to performing sequence analyses are familiar with identifying such indication of

common function at either the nucleotide level or peptide level, or both, including accounting for conservative amino acid changes. Such methods can be utilized in the present method. The computer-based sequence comparisons can be used to design complementary probes that can be used to isolate the actual coding and complete gene sequences.

Additional aspects and embodiments concerning thiaminases and thiaminase derivatives, and descriptions of the preparation and use of the same are provided in the related applications identified above, and are included within the scope of the present invention.

As used in the claims to describe the various inventive aspects and embodiments, "comprising" means including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Additional features and embodiments of the present invention will be apparent from the following Detailed Description and from the claims, all within the scope of the present invention.



### Detailed Description of the Preferred Embodiments

As indicated above, the present invention is related to the use of thiaminases as clinically useful reagents to induce apoptotic death of cells, including but not restricted to human tumor cells. It is based on the properties of an agent from the unicellular eukaryote *Naegleria gruberi* that induces delayed apoptotic death of mammalian cells, including tumor cells. These properties are summarized below, and more fully in the related Fulton et al. applications, *supra*, and in Lai et al. (paper in preparation).

Many chemotherapeutic and genotoxic agents, including radiation, that are currently used in cancer therapy share several unfavorable characteristics. Besides their considerable toxicity, these agents induce apoptotic death primarily in proliferating cells, leaving the temporarily quiescent cells found in solid tumors to re-grow. The ability to induce apoptosis in quiescent cells is of great therapeutic interest, since 1) in solid tumors many cells are quiescent, 2) commonly used chemotherapeutic agents mainly kill proliferating cells, and 3) most agents that induce apoptosis in quiescent cells are too toxic to use therapeutically ((Berges et al., 1995)). In addition, the ability of many commonly used agents to induce apoptosis depends on the expression of the *p53* tumor suppressor gene and is inhibited by expression of the *bcl-2* oncogene, yet many tumor cells survive because they are unable to produce functional *p53* protein or they overexpress *bcl-2*. The *Naegleria* agent, unlike common chemotherapeutic poisons, is not immediately cytotoxic but can induce apoptosis in quiescent, *p53* null, and *bcl-2*-expressing cells. The exceptional conditions under which this agent induces apoptosis suggest new opportunities for targeted cancer therapy.

The induction of apoptosis by the *Naegleria* agent is unusual in that the agent initially has little effect on the proliferation of the cells, and induces apoptosis only after a latent period of several days. With rat glioma C6 cells the time from Nex addition to death, the latent period, is about 4 days. The agent kills diverse mammalian cells, including primary cells and established cell lines of both normal and cancer cells. As the *Naegleria* agent is serially diluted a sharp end-point is reached after which cell death is no longer induced. The agent is very active; a  $10^{-6}$  dilution that contains 0.1  $\mu\text{g/l}$  of total *Naegleria* cell protein is sufficient to induce cell death. At the end of the latent period, death is rapid and extensive; within three

application of agents to induce a localized thiamin deficiency, it is always possible that an overdose could occur and produce more widespread effects than intended. With most chemotherapeutic agents, once the agent is introduced there is no ready antidote. A fortunate feature of our proposed therapies is that an effective antidote  
5 (i.e., thiamin) to thiamin-deficiency therapies is readily available, and can be applied quickly in large, nontoxic, inexpensive, easily administered doses, even after symptoms of thiamin deficiency appear. (Fulton et al., *supra*)

Many have reported the remarkably rapid recovery of animals and of people from thiamin deficiency, even after dramatic symptoms develop. For example,  
10 Peters describes the development of symptoms of thiamin deficiency in pigeons, up to convulsions and head retraction, and notes that these symptoms "could be cured in a matter of minutes when thiamin was injected into the subarachnoid space in the brain" (Peters, 1963). In humans, Platt describes how acute beriberi patients usually recovered within a few hours after being given a few milligrams of thiamin (Platt,  
15 1967). All these observations fit our *in vitro* result that cells can be brought to the brink of apoptosis by thiamin deficiency induced by thiaminase, to within hours of death, and yet show full recovery if excess thiamin is added to the culture medium.

The *Naegleria* thiaminase has been purified (Table 1, details below in Example 3) and shown to be a thiaminase I (EC 2.5.1.2), as the cleavage of thiamin  
20 is a base-substitution reaction dependent of a cosubstrate. Proof that the *Naegleria* apoptosis inducer functions as a thiaminase was obtained by cloning the gene and expressing it in *E. coli*. (see Example 3). The expressed protein had thiaminase activity that was effective at inducing apoptosis with the same latent period as the *Naegleria* agent. Apoptosis-inducing activity depends on the catalytic activity of the  
25 enzyme, since when we replaced Cys at the catalytic site of thiaminase I (see (Costello et al., 1996)) with a Ser, the replacement inactivated both the enzymatic activity and the ability of the protein to induce apoptosis (Table 2).

Thiaminase has no intrinsic toxicity to cell cultures, except due to depletion of thiamin (Lai and Fulton, unpublished data). In addition, early studies with  
30 animals showed that injected thiaminase of a mollusk remained in mice and rats without showing intrinsic toxicity, but caused a thiamin deficiency when administered parenterally for a week ((Ostrovsky et al., 1988; Puzach and Ostrovsky, 1976)).

Thiaminases are very effective at reducing thiamin concentrations, showing the amplifying power of a catalyst such that one thiaminase molecule can destroy many thiamin molecules. The *Bacillus thiaminolyticus* thiaminase I has a  $k_{\text{cat}}$  of 34  $\text{s}^{-1}$  (Costello et al, 1996). Our first estimates using the *Naegleria gruberi* thiaminase I expressed in *E. coli* have given a  $k_{\text{cat}}$  of  $\approx 100 \text{ s}^{-1}$  (N. Kuperwasser, E. Lai and C. Fulton, unpublished data).

We here present our *Naegleria* thiaminase gene sequence and the deduced amino acid sequence of its gene product (thiaminase protein) expressed in *E. coli* (Figs. 4–6). This expressed protein possesses thiamin-cleaving enzymatic activity and it is a potent death-inducing agent in its ability to induce apoptosis in vertebrate cells. In extracts made of the enzymes expressed in *E. coli*, the death-inducing activity of the *Naegleria* enzyme has been found to be 1000-fold higher than that of *Bacillus* thiaminase I; the extracts are active at dilutions of  $10^{-7}$  and  $10^{-3}$ , respectively. Some of this difference may be due to differences in the amount of enzyme produced in the two expression systems or to differences in the stability of the enzymes. As little as 50 pg/ml of partially purified, expressed *Naegleria* thiaminase was able to induce apoptosis of C6 cells in culture (see Example 2, below) in 4.5 days.

This report of the *Naegleria* thiaminase gene sequence provides us with a vector to transform live bacteria, which in turn can express the *Naegleria* agent, thiaminase I, that induces delayed apoptosis. Using methods familiar to those practiced in the art, these thiaminase gene products can be coupled to various ligands, or the coupling can be engineered at the DNA level and then expressed. The thiaminase can also be expressed in non-pathogenic bacteria designed to target to tumor sites (Example 6, below), where thiaminase can be utilized to induce apoptosis in the cancer cells. In addition, the finding of the expressed gene product allows us to mass-produce *Naegleria* thiaminase in *E. coli*. This thiaminase can then be coupled *in vitro* to tumor-specific antibodies or other elements (see Pat1) to be targeted to tumors *in vivo*. The genes can also be used in various methods of gene therapy.

## EXAMPLE 1. Assays for thiaminase activity

1-1. Measurement of thiaminase in liquid. Thiaminase activity was determined as described by others (e.g., (Costello et al., 1996)). In the case of thiaminase I, which depends on a base substitution reaction, a secondary nucleophile is used. The absorbance change at 252 nm, resulting from the reaction of thiamin with the secondary nucleophile (aniline), was monitored ((Lienhard, 1970)). The assay mixture contained 0.93 mM aniline, 0.093 mM thiamin, 100 mM sodium phosphate buffer (pH 6.5), and 2 mM dithiothreitol in a 1 ml volume at room temperature. The assay was initiated by addition of enzyme or sample and monitoring the change in absorbance at 252 nm at 0 min and 10 min. The activity was calculated according to the following equations ((Costello et al., 1996)) where  $A$  = absorbance change at 252 nm,  $V$  = total assay volume in microliters,  $\epsilon$  = the difference between the sum of the extinction coefficients of the products and the sum of the extinction coefficients of the reactants ( $11,200 \text{ M}^{-1} \text{ cm}^{-1}$  (aniline),  $2,415 \text{ M}^{-1} \text{ cm}^{-1}$  (veratrylamine)),  $l$  = path length of the cuvette,  $t$  = assay time, and  $p$  = protein in milligrams.

$$\text{Activity } (\mu\text{mol/min}) = (\Delta A)(V)/(\Delta \epsilon)(l)(t).$$

$$\text{Specific activity } (\mu\text{mol/min/mg}) = (\Delta A)(V)/(\Delta \epsilon)(l)(t)(p).$$

One unit of thiaminase is defined as the amount of enzyme that will produce 1  $\mu\text{mol}$  of product per min at  $25^\circ\text{C}$ .

Other methods of assay are available. For both thiaminase I and II, one can also measure the amount of thiamin that remains using the thiochrome method (see Wyatt et al., 1989), or measure the degradation directly using thiamin that is radioactively labeled in the thiazole ring (Evans, 1975; Edwin, 1979; Alston and Abeles, 1987).

1-2. Visualization of thiaminase on a solid support. Detection of thiaminase on solid supports, such as agar or polyacrylamide gels, can be achieved by a simple staining procedure which involves detection of residual thiamin by a colorimetric assay using a diazo coupling reagent ((Abe et al., 1987)). Diazo reagent was prepared as described ((Abe et al., 1986)). Wherever thiamin is present the gel support turns pink, whereas where thiamin is absent (e.g., destroyed by thiaminase), the gel is colorless.

The following protocol is specifically for detection of thiaminase following polyacrylamide gel electrophoresis. The gel after electrophoresis and subsequent wash was placed in a solution of 1 mg/ml thiamin, 1 mg/ml aniline (Fisher, distilled, 1 mg=0.978  $\mu$ l), 2 mM dithiothreitol, and 25 mM sodium phosphate buffer (pH 6.5) at room temperature for 10 min, and then the solution was discarded. Residual excess of the solution on the gel surface was absorbed with a filter paper and incubated at 37°C for 30 min in a covered box. The degradation of thiamin was detected by the diazo coupling reaction as described below.

The diazotized p-aminoacetophenone mixture was poured on the gel that had completed the incubation as described above (16 ml for 5 min, then an additional 16 ml for another 5 min, against 26 cm<sup>2</sup> of gel surface). After 10 min at room temperature, the solution was decanted. The diazo group couples to the thiamin and turns the background of the gel into a reddish-pink color. When thiaminase catalyzes the substitution reaction between thiamin and aniline, the diazo reagent is not able to couple to the free thiazole or the substituted base covalently linked to the pyrimidine ring, resulting in a "white halo" on the pinkish gel.

#### EXAMPLE 2: Functional test for apoptosis-inducing activity *in vitro*

Many cell lines can be utilized for assay of the apoptosis-inducing activity of thiaminase. The following protocol describes the use of C6 rat glioma cells, but would be equally applicable, e.g., to HeLa human cervical carcinoma cells.

C6 rat glioma cells were plated at  $1 \times 10^5$  cells/ml in medium 199 containing 10% fetal bovine serum and 50  $\mu$ g/100 ml gentamicin and a dilution of agent or extract to be tested was added at the time of plating, designated day 0. Assays were conducted under various conditions, e.g., 1 ml per well in a 24-well multiwell dish. The cultures were observed at ~12 hour intervals. The latent period was scored by noting when cell death created about 25% open space in the culture, as readily evaluated visually. and the time of apoptosis noted.

*Naegleria* cell extracts were prepared by harvesting and resuspending the cell pellet in 10 volumes of sterile demineralized water, followed by three freeze-thaw cycles, centrifuging the suspension, and filtering the supernatant through a 0.22  $\mu$ m pore size Millipore filter. Dilutions of cell extracts continue from this  $10^{-1}$

solution. Dilutions of cell extracts were added to each well. Cells were allowed to grow at 37°C in 95:5 air:CO<sub>2</sub>.

EXAMPLE 3: Purifying thiaminase from *Naegleria*, cloning and expressing its gene, and testing for enzyme activity and apoptosis-inducing activity

5

Purification of thiaminase.

The strategy used for purification of *Naegleria* thiaminase, with the results of a sample purification, is outlined in Table 1. The original preparation of the *Naegleria* apoptosis-inducing agent used three cycles of freeze-thaw ((Dunnebacke and Schuster, 1971)); this procedure was modified for preparation of *Naegleria* extract, Nex, and as a first step in the purification of the apoptosis-inducing agent.

3-1. Freeze-thaw. Sixty baking trays of *Naegleria gruberi* strain NEG ((Fulton, 1970)) amebae ( $6 \times 10^{10}$  cells) ((Kowit and Fulton, 1974)) were harvested, washed three times by centrifugation ( $900 \times g$  at 4°C for 1 min) and centrifuged into a pellet, with all supernatant decanted. These pellets could be prepared from two batches of 30 trays each and stored at -70°C until use. The cell pellets were suspended in 10 volumes of Dialysis Buffer (50 mM Tris.HCl, pH 7.5, 2 mM dithiothreitol, 2 mM EDTA (ethylenediaminetetraacetic acid)) and subjected to four cycles of freeze-thaw at -72°C (dry ice-ethanol) and 22°C (water bath). The lysate after the freeze-thaw step was centrifuged for 30 min at 4°C at 9000 rpm ( $6000 \times g$ ) using a Beckman JA-20 rotor. The supernatant was used for the next step.

3-2. Ammonium sulfate fractionation. The supernatant was adjusted to 50% saturation by the slow addition of solid ammonium sulfate (to 291 g/l) at 0°C, stirred for 1 h at 0°C and centrifuged at 11000 rpm ( $9500 \times g$ ) at 4°C for 20 min. The supernatant was then brought to 70% saturation by the addition of solid ammonium sulfate (an additional 125 g/l) at 0°C and stirred for 1 h before centrifugation at  $9500 \times g$  for 20 min at 4°C. The resulting pellet of material soluble in 50% but insoluble in 70% ammonium sulfate was dissolved in 10–24 ml of Dialysis Buffer (using the smallest volume in which the entire sample appeared to dissolve), and dialyzed in the same buffer in the cold room overnight with two changes of buffer, 1 liter each.

3-3. DEAE fractionation. The dialyzate was filtered using first a 0.45  $\mu$ m Millex-HA sterile filter (Millipore Corp.) and then a 0.22  $\mu$ m Millex-GV sterile filter. The sample was loaded onto a DEAE-ion exchange (Whatman DE 52 pre-swollen microgranular anion exchanger diethyl aminoethyl cellulose, catalogue no. 4057-050) column (Glenco 32 cm X 28 mm (inside diameter)) equilibrated with Dialysis Buffer at a pump flow rate of 1.4 ml/min using LKB VARIOPERPEX® II. The column was washed thoroughly with the same buffer to remove unbound protein. The protein was eluted using a sodium chloride step gradient of 0 to 0.3 M NaCl. The fractions containing thiaminase activity were pooled, and concentrated by ultrafiltration (Amicon, PM-10) (Amicon, Beverly, MA 01915). The protein was dialyzed in a BioCad Buffer (20 mM Tris/BIS-propane [80% pH 6.0, 20% pH 9.0], 2 mM dithiothreitol) at 4°C overnight with two changes of buffer, 1 liter each.

3-4. PerSeptive high-speed perfusion (column) chromatography. The dialyzed sample was loaded onto a 4.6 mm diameter, 100 mm long column containing a POROS® 20 HQ Media packed according to manufacturer's instructions and fractionated using a BioCAD™ SPRINT™ Perfusion Chromatography® System and an ADVANTEC SF-2120 Super fraction collector. The fractions containing thiaminase activity were pooled and dialyzed against 2 mM dithiothreitol in the cold room overnight with 2–3 changes of buffer, 1 liter each.

3-5. Preparative IEF (isoelectric focusing). The sample was further purified using the technique of preparative IEF, which utilizes a Bio-Rad Rotofor (model 3000Xi computer controlled electrophoresis) and Bio-Rad RotoLyte pH range 3.9–5.6. The fractions containing thiaminase activity were pooled and dialyzed in Tris/DTT buffer (50 mM Tris.HCl, pH 7.5, 2 mM dithiothreitol) in the cold room overnight with 2 changes of buffer, 1 liter each. The dialyzed sample was concentrated using a Microcon-10 (10,000 MW cut-off) (Amicon).

3-6. Laemmli SDS-polyacrylamide gel electrophoresis. The partially purified sample of thiaminase after the preparative isoelectric focusing step was further analyzed by Laemmli SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel. Duplicate sets of samples including protein size standards were run on one gel. After electrophoresis, one-half of the gel was stained and destained. The other half of the gel was washed with 200 ml of 25 mM sodium phosphate buffer (pH 6.5) with slow stirring to remove SDS at room temperature with two

changes of buffer for 20 min each. The thiaminase band(s) on the gel was detected colorimetrically using a diazo coupling reagent (Example 1–2, above). An example of this visualization is shown in Fig. 3.

To visualize protein bands, the gel was stained after the diazo coupling  
 5 reaction with 0.1% Coomassie blue R250 in water/methanol/acetic acid (5:5:2). This procedure allowed the verification of the band with thiaminase activity to be stained by a protein stain.

Although precise means to determine the amount of thiaminase I enzyme in  
 10 *Naegleria* are not yet available, on the basis of the amount recovered during purification and the documented losses (Table 1), we estimate that thiaminase I accounts for about 0.01% of the total protein of the amoebae.

The band of protein with thiaminase activity was excised from the gel, rinsed with 50% acetonitrile, and sent to Harvard Microchemistry Facility (Harvard University, Cambridge, MA) for microsequencing in order to obtain sequences of  
 15 peptides to seek and identify the thiaminase gene.

3-7. Microsequencing. The protein was enzymatically digested and analyzed by HPLC chromatography. Peptides were selected and further analyzed by matrix-assisted desorption time-of-flight mass spectrometry (MALDI-TOF MS) performed on a PerSeptive BioSystems Voyager-DE STR (Framingham, MA) ((Chait and  
 20 Kent, 1992)). These peptides were sequenced with high confidence as supported by MALDI result.

### Cloning the *Naegleria* thiaminase gene

3-8. Genomic clone isolation. Three peptide fragment sequences derived from analysis of the gel purified putative thiaminase were obtained. Peptides A  
 25 (ASDLPQSGDQVNK), B (TILDSTVVASQR), and C (SSNFYAQLSQQFDAK) were compared to the peptide sequence of *Bacillus subtilis* thiaminase I precursor to determine the best estimated local alignment for each fragment using LALIGN (FASTA v.2.0u54 (X. Huang and W. Miller (1991) Adv. Appl. Math. 12:337-357)). Sets of forward and reverse degenerate oligonucleotide primers were designed based  
 30 on these alignments, and synthesized using an Expedite™ model 899 DNA synthesizer using phosphoramidite synthesis chemistry (PerSeptive Biosystems, Framingham, MA). Peptide A forward primer AF1 (5'-CARWSIGGGHAYCARG-



- 3') and peptide C reverse primer CR1 (5'-TTIGCRTCRAAYTGYTG) generated an  
 amplicon of ~400 bp as expected based on the *B. subtilis* alignments, was  
 designated AC400 and chosen for gel purification using a QIAEXII gel purification  
 kit (QIAGEN, Santa Clarita, CA). Fluorescent DNA sequencing reactions using the  
 CR1 degenerate oligonucleotide and the gel purified AC400 PCR DNA fragment  
 were performed using an Applied Biosystems 373 Stretch DNA Sequencer, and ABI  
 PRISM™ dye terminator cycle sequencing reagents using DNA polymerase FS  
 following manufacturer recommendations (PE Biosystems, Foster City, CA). The  
 resulting sequence facilitated the design of two homologous internal  
 oligonucleotides a forward primer AC1 (5'-TGTCGGATATAGTGAAAGTATG-  
 3') and a reverse primer AC2 (5'-AACCTTTTGCTTTTCATCACAC-3'), which  
 were used to complete the 431 bp DNA sequence of AC400 that encodes a peptide  
 with strong homology to *B. subtilis* thiaminase I precursor. AC400 was labeled  
 using the random hexamer primer method and used to screen a *Naegleria gruberi*  
 strain NEG λEMBL3 genomic DNA library ((Lai et al., 1988b)), with each of the  
 positive clones sequenced with oligonucleotides AC1 and AC2. The resulting  
 genomic clone φTTK containing all of the 431 bp of the AC400 hybridization probe  
 was chosen for more extensive DNA sequence analysis using oligonucleotide  
 primers.
- 3-9. Sequence analysis and expression cassette construction. Analysis of  
 3414 bp of genomic sequence from φTTK defined an ORF of 3078 bp (Fig. 4)  
 encoding a 1025 amino acid peptide of MW 112,141 daltons (Fig. 5), with the  
 amino terminal domain amino acid residues Met1 to Leu356 encoding a peptide of  
 MW 39,079 daltons having strong homology to *B. subtilis* thiaminase I precursor.  
 This homology is shown in Fig. 8, as well as the positions of the three peptide  
 sequences from *Naegleria* thiaminase that were used to find the gene. This region  
 was chosen for expression in pET-22b(+), with the expression cassette generated  
 by PCR amplification of φTTK target DNA using *pfu* polymerase (STRATAGENE,  
 La Jolla, CA). An NdeI site was introduced at the ATG start codon using a forward  
 primer CDA1 (5'-GAGATATACATATGTCCACTCAACCAAAGAC-3'), and a  
 TAA stop codon followed by a BamHI site was introduced after Leu356 using the  
 reverse primer CDA2 (5'-TATGGATCCTTAAAGGAA

TGGTCTCAAGACACC-3'). The resulting 1091 bp amplicon was digested with NdeI and BamHI, gel purified and the expression cassette ligated into pET-22b(+) followed by transformation into *E. coli* XL1-blue™ (STRATAGENE, La Jolla, CA). Sequencing of the resulting plasmid pNB1+ verified the expected 1068 bp ORF (Fig. 6) including the potential catalytic Cys118, as well as the junctions essential for expression of the 356 amino acid Met1–Leu356 peptide (Fig. 7).

### Expressing *Naegleria* thiaminase in *E. coli*

3-10. Expression and functional selection. Expression and functional selection were based on enzymatic activity of colonies on agar (Example 1). The recombinant pET-22b(+) plasmid (Promega, Madison WI), containing the 1068 bp (base pair) NdeI-BamHI fragment encoding the *Naegleria* thiaminase gene sequence was isolated and purified from *E. coli* XLI-blue™ strain, and subsequently used to transform *E. coli* BLR(DE3)pLysS (Novagen), or BL21(DE3)pLysS (Novagen). The positive clones were selected on agar by the same diazo coupling reagent as discussed above (see Section 3-6). This assay is based on the abilities of the clones to synthesize functional thiaminase that could cleave thiamin in situ on agar (Abe+86). These positive clones were further confirmed by preparing freeze-thaw cell extracts and assaying for their thiaminase I activity using the spectrophotometric method mentioned above (see Example 1-1) and by performing a cell death assay on rat glioma C6 cells (Example 2). A clone that produced a high level of thiaminase was designated pNB1+.

### Site-directed mutagenesis of thiaminase I

3-11. PCR site-directed mutagenesis. PCR site directed mutagenesis of the potentially catalytic cys118 codon (TGC) to ser118 (AGC) was performed using a variation on the method of Baretino et al. (1994) N.A.R. 22(3):541-542. The position of the catalytic Cys codon is shown in Fig. 8. A mutagenic coding strand (reverse) primer CDA3 (5'-CAA TAA AAA GTT TGA GCT CAA GTA TTG-3') was used in conjunction with the forward primer CDA1 to amplify a 380 bp mutagenic megaprimer. Following gel purification the megaprimer was extended using the reverse primer CDA2 in a linear PCR extension reaction. The resulting 1091 bp fragment, containing a GCT (encoding Ser) instead of GCA (encoding Cys118) was digested with NdeI and BamHI, subcloned, sequenced and expressed

SD-131631.1

[illegible]

#### EXAMPLE 4. Cloning and sequencing of other thiaminase genes

The cloning and sequencing of other thiaminase genes can be accomplished by one or more of the following methods. The methods are listed in the order of the ease of their performance. If the first method proves not applicable, the second method should be tried, and so on.

*Naegleria* thiaminase I, as isolated in *Naegleria* extract and as expressed in active form in pNB1+ (Figs. 6–7), has a molecular weight of  $\approx 40,000$ , similar to the *Bacillus thiaminolyticus* enzyme, which is 42,000 ((Costello et al., 1996)). A comparison of the sequences of these two enzymes is given in Fig. 8. The normal isolation protocol for the *Naegleria* enzyme involves repeated cycles of freezing and thawing. This protocol also releases all the proteases of this “bacteria-eating” amoeba, and in our extensive experience with this organism few proteins survive this harsh procedure. The survival of *Naegleria* thiaminase I to repeated freeze-thaw cycles is an indication of the remarkable stability of this protein to proteolytic enzymes, a useful attribute for potential therapeutic use *in vivo*. In *Naegleria* the thiaminase is encoded as segment of a protein of  $\approx 110$  kDa (Figs. 4–5). We have been able to detect this protein, with thiaminase activity, using protocols other than freeze-thaw to lyse the amoebae. The function of the thiaminase portion of the protein in living *Naegleria* (or any organism) is quite unknown. It is unlikely that thiaminases are active in living cells ((Fujita, 1954)), and it is even possible that this segment possesses a function distinct from cleaving thiamin, especially when in association with the rest of the encoded protein. Mollusks and ferns also have thiaminase I proteins whose molecular weights have been estimated at 93–100 kDa ((McCleary and Chick, 1977)), so in other eukaryotes it is possible that the entire protein, and not just the segment encoding thiaminase I, may prove homologous to the *Naegleria* protein.

##### 4-1. Homologous sequences

Based on the literature, sequences obviously homologous to the *Naegleria gruberi* strain NEG thiaminase I sequence can be expected to be readily found in,

and obtainable from, organisms including: other species and strains of *Naegleria*; certain plants, especially ferns such as bracken and nardoo; some molluscs, such as clams; certain fish, especially carp, and certain bacteria other than *Bacillus thiaminolyticus*. No sequences from any such organisms have been characterized, but based on available information, and comparison of the *Naegleria gruberi* and *Bacillus thiaminolyticus* sequences (Fig. 8), these thiaminases are anticipated to be clear homologues. It is advantageous to have isolated, purified, or enriched nucleic acid sequences encoding such thiaminases for use in the present invention. Such nucleic acid sequences can be obtained using routine techniques known to those skilled in the art.

We claim as homologues any protein, and any gene that encodes a protein, with the following characteristics, and any derivative of such a protein useful in relation to apoptotic therapies:

1) Thiaminase I activity in its wild-type form. Mutation *in vivo* or *in vitro* to catalytically inactivate the protein does not remove the protein or its genes from homologous status.

2) Overall similarity of the amino acid sequence over the entire length of the thiaminase I protein, or the thiaminase I segment of a larger protein. This similarity can be established by comparing the thiaminase I encoded by pNB1 (Fig. 7) with the overall amino acid sequence in question using the FASTA program ((Pearson and Lipman, 1988)). Currently, in September 1999, using the amino acid sequence of *Naegleria* thiaminase I (Fig. 5) to search the non-redundant protein library (NCBI database), with default parameters, yielded the *Bacillus* thiaminase I with  $\approx 25\%$  identity in a 358 amino acid overlap. No other proteins that showed strong identity over a long overlapping sequence. A suitable criterion for a putative thiaminase would be  $>22\%$  identity over 300 amino acids.

3) Local similarity to active site regions as determined by BLAST. For example, the catalytic domain segment of 13 residues surrounding the active site Cys, VYGFPQYLCSNFL, would be expected to give an identity of 8 of the 13 amino acids (see Fig. 8).

Once candidate thiaminases are recognized by these criteria, they can be expected to show other features. One example is the six amino acid sequence GYSESM that starts at residue 228 of *Naegleria* thiaminase I (Fig. 7). This is part of

the pyrimidine coordinate residues, and one would expect a match of  $\geq 5$  of the 6 residues.

- [Wes: these criteria include rather than exclude *Bacillus thiaminase*. Do we need more stringent criteria? If so, we have to re-define so different from *Bacillus*. See Pat 1, p. 33, l. 27, etc.]

#### 4-2. Direct PCR method

- 10 In one approach, using procedures known to those skilled in the art, the amino acid sequence can be used to design DNA primers, and these can be used in conjunction with the polymerase chain reaction (PCR) to identify the corresponding thiaminase gene (genomic or cDNA sequences) (Mullis et al., 1994). Preferably, amino acid sequences are used which correspond to unique or low degeneracy primer
- 15 sequences. Genes will be cloned, sequenced, and expressed using standard techniques (Sambrook et al., 1989). Total DNA is prepared from the organism whose thiaminase gene one wishes to clone. Standard methods known to those experienced in the art can be used ((Sambrook et al., 1989)), and specific methods have been developed for plants and especially for ferns ((Dempster et al., 1999)).
- 20 Oligonucleotide primers will be designed based on our knowledge of *Naegleria* thiaminase I gene sequence (Fig. 6-7), comparison with the *Bacillus thiaminolyticus* sequence (Fig. 8), and knowledge of codon-usage patterns in the organism in which the gene is being sought. These primer sets will be used to prime PCR reactions in order to locate the gene of interest. Additional oligonucleotide primers will be
- 25 prepared to allow extensive sequencing of the whole gene, as well as cloning it (see example in Sections 3-8 to 3-10).

#### 4-3. Using the *Naegleria* thiaminase gene as a heterologous probe

- The *Naegleria* thiaminase gene can be used as a heterologous DNA probe to screen for thiaminase genes in other organisms. Total genomic DNA will be
- 30 prepared from any organisms to be screened for thiaminase (as in Section 4-2, above). To determine if there is sufficient homology between the *Naegleria* thiaminase gene and those being sought, a pilot Southern blot experiment will be performed. It is recognized that the apparent homology can be affected by introns,

which are absent in the *Naegleria* thiaminase gene but may be present in others. The total genomic DNA is digested with restriction enzymes, the digested products are run on a DNA agarose gel, then transferred onto nitrocellulose ((Southern, 1975)). The *Naegleria* thiaminase gene is  $^{32}\text{P}$ -labeled by random hexamer primer labeling and used as a probe to hybridize to the DNA on the nitrocellulose using non-stringent hybridization conditions we developed ((Lai et al., 1988a)). A positive signal on the autoradiograph would indicate that sufficient homology exists between the *Naegleria* gene and the desired gene. A phage lambda genomic library would be constructed using total DNA of the organism of interest. The *Naegleria* thiaminase gene will be used as a heterologous probe to screen these libraries for the desired thiaminase gene.

#### 4-4. cDNA expression cloning

Total mRNA will be extracted from the organism of interest, and a cDNA expression library constructed using standard procedures (as (Sambrook et al., 1989). Colonies expressing the thiaminase gene will be sought using the diazo reagent method in agar (see Sections 1-2 and 3-10).

#### 4-5. Purification of thiaminase protein followed by microsequencing of the proteins, cloning, and DNA sequencing

Thiaminases can be purified by following the methods described in the literature (see Fulton et al, *supra*, for an extensive review), as well as our more current methods described in detail here (Sections 3-1 through 3-7). Thiaminases can also be purified using standard methods used by other workers (Deutscher, 1990; Menge, 1994; Costello et al., 1996). In early studies, fish thiaminase ((Ågren, 1945; Ågren, 1946)), clam and mussel thiaminases ((Alston and Abeles, 1987; Fujita, 1955; McCleary and Chick, 1977)), fern thiaminases ((Kenten, 1957; McCleary and Chick, 1977)), and thiaminases of *Clostridium sporogenes* and *Bacillus aneurinolyticus* ((Ikehata, 1960; Kobayashi, 1975; Wittliff and Airth, 1970)) have been partially purified. Purification can be monitored by assays of thiaminase activity (Section 1-1 and 1-2), evaluations of protein purity by gel electrophoresis (Section 3-6), and assays of death-inducing activity (Example 2). Once the purified thiaminase protein is available, it will be microsequenced to obtain partial peptide information (as Section 3-7). Oligonucleotides will be designed and prepared based on peptide sequence information, and used for screening a genomic DNA or a cDNA library (as

Section 3-8). The resulting positive clone(s) will be sequenced to confirm that it encodes a thiaminase (as Section 3-9). These clones will subsequently be expressed in *E. coli* and the expression clones selected using the diazo coupling reagent in agar (see Section 3-10). Cell extracts will be prepared from these clones and the

5 thiaminase enzyme activity will be determined as described (Example 1). Cell death assays will be performed as described (Example 2).

#### EXAMPLE 5. Targeting thiaminase for localized delivery

Effective therapeutic use of thiaminase is likely to require localizing the thiaminase to target cells or tissues, since an overall thiamin deficiency would cause the various

10 symptoms of beriberi. Many means are available for targeting thiaminase, as detailed in Fulton et al., *supra*. These include the following examples, all described in detail in Fulton et al., *supra*, all familiar to those in the field of cancer therapy:

- 1) Localized administration, e.g., upstream of a capillary bed feeding a tumor.
- 15 2) Targeting by coupling thiaminase to an antibody or an antibody derivative that will carry it to the desired cells or tissues.
- 3) Targeting by coupling thiaminase to a targeting receptor ligand.
- 4) Targeting by binding thiaminase to a liposome or other delivery vehicle that will target it to the desired cells.
- 20 5) Targeted gene therapy using thiaminase, e.g., using a hypoxic response element that will express the thiaminase gene in the hypoxic regions of solid tumors.
- 6) Target using nonpathogenic bacteria to see tumors and there express thiaminase.
- 7) Making the enzyme inactive until localized, by depending on a property
- 25 that would activate the enzyme in the target tissue (such as using the protease activity of prostate specific antigen to turn on the enzyme in prostate tumor tissue).
- 8) Target the enzyme (e.g., by using bacteria as a warhead) to specific cavities in the body, such as the bladder or the colon.
- 9) Target to the lung by pulmonary absorption.
- 30 In connection with these various targeting techniques, those skilled in the art will recognize that such targeting can be utilized for both the administration of



thiamin-depleting agents and for the administration of nucleic acid sequences encoding thiamin-depleting agents.

EXAMPLE 6. Thiaminase carried by non-pathogenic bacteria used for prostate cancer therapy

- 5 It is instructive to consider one example of specific targeting of thiaminase for therapy of a specific cancer. The example chosen is the use of thiaminase carried as a warhead on a non-pathogenic bacteria that will seek and attack metastatic prostate cancer. It will be evident that this example could easily be applied to other types of cancer and to other methods of localizing thiaminase or its genes.
- 10 Prostate cancer is responsible for 43% of new noncutaneous cancer cases in men, and has become the second leading cause of male cancer deaths (da Vita, 1997, pp. 1322-1386). The tumor cells in the adenocarcinomas are initially androgen dependent, and androgen ablation (e.g., castration) causes apoptosis of these cells. This causes initial remission of the prostate cancer, but any tumor cells left behind
- 15 eventually change from androgen-dependent to androgen-independent (see Umekita et al., 1996). Thus after an initial remission as the androgen-dependent cells die, within a year or two the androgen-independent cancer cells become evident, grow, metastasize, and eventually kill the patient. The major problem in devising a therapy to kill these androgen-independent, metastatic tumor cells has been that
- 20 most of the cells are not actively proliferating at any particular time; it has been estimated that about 2% undergo division on any given day. As J. T. Isaacs and his coworkers put it, "Unfortunately, more than 90% of prostatic cancer cells within an individual patient are in interphase" (Kyprianou et al., 1991). These authors concluded that the only hope to improve survival rates for prostate cancer is
- 25 simultaneous therapy of androgen ablation to kill the androgen-dependent cells and some therapy to eliminate the androgen-independent cells. One way to eliminate the androgen-independent cells is to increase the rate of apoptosis among non-proliferating cancer cells.

- 30 Prostate cancer provides an excellent situation for use of thiamin-depletion-induced apoptosis. The many cells lines in which the *Naegleria* agent (thiaminase I) was shown to induce apoptosis include two cell lines derived from human prostate cancers, LNCaP, which is an androgen-dependent cell line with a functional *p53*

gene, and PC-3, which is an androgen-independent cell line that is null for the *p53* gene. Thus, prostate cancers are among good candidates for treatments using our invention. In order to induce apoptosis in these cancer cells, it would suffice to surround the cells, the tissues, or the tumors with thiaminase, and thereby starve the  
5 cells for thiamin.

For this therapy it is important to maintain the low level of thiaminase continuously around the cancer cells until the cells become depleted of the vitamin and undergo apoptosis. The therapy does not require that every cell be surrounded. Adequately surrounding a group of cells (e.g., a solid tumor) would be sufficient. A  
10 particularly useful approach would be to localize the thiaminase by lining the walls of the capillaries that feed the prostate cancer cell mass, thus destroying all thiamin brought to the tissue. The principle of this method is to surround the cancer cells and starve them of thiamin until they all undergo apoptosis. Properly applied, this therapeutic approach could avoid the widespread nonspecific tissue damage that  
15 accompanies use of poisons and radiation. While one could use antibodies or other targeting ligands to carry the thiaminase to the cancer, there are advantages to using non-pathogenic bacteria.

Bacterial infections of tumors, sometimes associated with regression of the tumors, have been known for two centuries ((Nauts et al., 1953; Wiemann and  
20 Starnes, 1994)). There have been many reports of the use of living bacteria for cancer therapy (reviewed in Minton and Oultram, 1988; Pawelek et al., 1997; Saltzman et al., 1997). In one frequent use, since 1976, attenuated "bacille Calmette-Guérin" (BCG) has been instilled intravesically into bladders to reduce recurrence of bladder cancer. The procedure offers benefit (e.g., Lamm et al., 1991).

25 One especially notable approach involves the use of *Salmonella* spp., especially *S. typhimurium*, for cancer therapy (e.g., (Low et al., 1999; Pawelek et al., 1997; Saltzman et al., 1997)). For example, it was found (Pawelek et al., 1997) that attenuated (non-pathogenic) hyperinvasive, polyauxotrophic mutants of *S. typhimurium* targeted melanomas in mice, and *in vivo* reduce the rate of tumor  
30 growth and increased survival to as much as twice the survival time of uninfected mice. The bacteria were found at high concentrations in the tumors, both in the necrotic zone of the tumors and inside cells.

The advantageous attributes of *Salmonella* as a living vector to deliver thiaminase to a tumor include:

1. known affinity of *Salmonella* for tumors, and growth therein;
2. presence on these bacteria of systems for invading vertebrate cells;
- 5 3. ease of culture;
4. facultative anaerobe, able to grow both aerobically and anaerobically, whether in culture or in tumors;
5. ease of isolating mutants, e.g., attenuated, hyperinvasive, or auxotrophs;
6. availability of the extensive, powerful genetics and molecular biology techniques of enteric bacteria; and
- 10 7. extensive knowledge of the pathogenicity of this species.

To use this system for thiamin-deficiency therapy, attenuated, hyperinvasive bacterial strains that efficiently express and secrete thiaminase are engineered, using well known molecular biology techniques. These bacteria would be injected into  
 15 animals bearing tumors, where they would preferentially locate and grow in the tumors, both intracellularly and extracellularly. The thiaminase produced by those bacteria would cause LAIDT. As with other methods of causing LAIDT, this treatment can be combined with other therapies.

Another example of the use of bacteria as tumor-specific, amplifiable protein  
 20 expression vectors is the use of an anaerobe to specifically target the hypoxic environment of tumors. While cells at the surface of a tumor mass often are actively proliferating and usually susceptible to radiation and chemotherapy, the central cells, in addition to being inaccessible, are largely not proliferating and stubbornly resistant to therapeutic treatments (Hickman et al., 1994). The centers of such solid  
 25 tumors are often hypoxic (oxygen deficient), an environment that favors selection of cells that express bcl-2 but not p53 and thus become highly resistant to apoptosis induced by currently used therapeutic agents (Graeber et al., 1996). Hypoxia in these tumor masses is correlated with insensitivity to non-surgical therapies and a poor prognosis for successful control of the cancer and for patient survival. The  
 30 presence of hypoxic tumors was a strong predictor of probable disease recurrence and of poor survival (Höckel et al., 1996).

Based on *in vitro* studies, it is anticipated that sustained thiamin depletion will induce apoptosis in the non-cycling "dormant" cells of solid tumor masses. In

addition to the possibility of “surrounding” tumor masses with a thiamin-cleaving compound, another approach to LAIDT uses anaerobic bacteria as carriers.

Spores of *Clostridium* species have long been known to exhibit remarkable specificity for solid tumors. The spores become localized in, germinate, and grow in the hypoxic tissue (reviewed in Minton et al., 1995). This unique property can be used to locate solid tumors in the body as well as to target therapeutic agents. Even nonpathogenic species kill some of the cancer cells, but infection alone is not sufficient to kill the tumor. For example, *C. butyricum* M-55 injected intravenously or intratumorally caused tumor lysis after 5–8 days, in some cases leaving only the outer rim of cancer cells (which regrew) (e.g., Möse and Möse, 1959; Heppner and Möse, 1978). Any associated toxemia could be controlled with antibiotics.

Recently, avirulent *C. beijerinckii* have been genetically engineered to express enzymes that would cleave prodrugs (Minton et al., 1995; Fox et al., 1996), and techniques for genetic engineering of Clostridia have been developed (Rood et al., 1997). Animals bearing solid tumors are injected with these Clostridia, and, after the bacteria have lodged in the tumors, the animals are injected with the prodrug. Cleavage of the prodrug by the enzyme secreted in the tumor targets the toxic drug to the core of solid tumor.

*Clostridium* sp. that expresses thiaminase can be used to target and infect solid tumors, and thereby induce localized thiamin deficiency. One possibility for a suitable bacterium would be the avirulent *C. sporogenes* ATCC 8075, already known to make and secrete a thiaminase I (Kobayashi, 1975a). The *C. butyricum* M-55 used in early attempts to treat tumors is now classified as *C. sporogenes* ATCC 13732, so is presumably closely related to the strain known to secrete thiaminase. An alternative possibility is to use another species of *Clostridium*, or even another genus of anaerobes. It may be desirable to genetically restrict or attenuate the bacterium used so it will accomplish the effective delivery of thiaminase and induction of apoptosis without causing unwanted sepsis. One possibility would be to use the *C. beijerinckii* currently being used in prodrug experiments (Minton et al., 1995; Fox et al., 1996), and to engineer this strain to overexpress and secrete an appropriate thiaminase.

Treatment of solid tumors with Clostridia expressing thiaminase would induce apoptosis of the central tumor mass. To assure death of the whole tumor

mass the *Clostridium*-thiaminase therapy would preferably be coupled with a treatment directed at the peripheral cells, such as conventional radiation and chemotherapy or separately surrounding these cells with thiamin-cleaving compound by some other means, for example, using an antibody-thiaminase conjugate.

#### EXAMPLE 7. Combination of thiamin depletion and accessory treatment for targeted cells

In addition to the use of thiamin depletion to induce apoptosis of a targeted group of cells, a variety of different accessory treatments can be utilized in conjunction with the thiamin depletion to enhance the effectiveness of the treatment. Conversely, for some treatments, such as other anti-neoplastic treatments, the use of apoptosis induced by thiamin depletion can be regarded as enhancing the effectiveness of that other treatment. Thus, the present invention includes such combination therapy, where thiamin deficiency induced apoptosis is used in conjunction with another treatment method. In these combination methods, the thiamin depletion can be targeted by any of a variety of methods, for example, by any of the methods described herein. Also, the accessory methods also preferably involve localized or targeted treatment. Any of a variety of methods may be used for such targeting also, again for example, the targeting methods described for targeting of thiamin depletion, with the choice of targeting method being appropriate for the type of composition involved in the accessory treatment. Those skilled in the art will readily recognize the appropriate selections, which include a variety of standard treatments involving radiation and chemotherapy.

One particularly promising therapy to combine with LAIDT is antiangiogenesis. It has been shown that the growth of solid tumors beyond about 2 mm depends on vascularization (Folkman, 1997). Recent studies have shown that inhibitors of this angiogenesis can result in tumors becoming quiescent (Boehm *et al.*, 1997; O'Reilly *et al.*, 1996; Skobe *et al.*, 1997). As described above, thiamin deficiency induced apoptosis kills quiescent cells. Thus, inducing quiescence of cells in a solid tumor would markedly enhance the therapeutic effectiveness of inducing a targeted thiamin deficiency in that tumor by increasing the fraction of quiescent cells.

Antiangiogenesis therapy appears to work by disrupting the tumor microvasculature that provides nutrients, including oxygen, to a tumor and takes away waste. In that sense, antiangiogenesis therapy produces overall "starvation" of the tumor. Some cells die, apparently both by necrosis and by apoptosis, while others simply become dormant (quiescent). *Overall* starvation, depending on the exact (and locally variable) conditions, induces death of some cells and quiescence in others. In contrast, *specific* starvation for thiamin induces apoptotic death, even in quiescent cells. Thus, these paradigms differ, and are, in fact, complementary. Used together, for example, antiangiogenesis therapy can arrest cell growth and LAIDT therapy can kill the arrested cells.

A variety of antiangiogenesis compounds were described in Fulton et al., *supra*, and can be utilized herein.

#### EXAMPLE 8. Use of inactive thiaminase and multiple thiaminases to avoid rejection

As a foreign protein, it is anticipated that thiaminase may cause an immune reaction. In previous experiments, we have shown that injection of rabbits with concentrated extracts of *Naegleria*, which we now know contained the *Naegleria* agent (thiaminase I), did not cause sickness or death, although the injections did elicit antibodies to *Naegleria* proteins (Fulton, 1970, p. 454). There is no reason to anticipate that thiaminase would be toxic to animals except by lowering the thiamin concentration. In time, however, it is likely that circulating thiaminase would lead to production of antibodies against itself. Immunosuppression and other methods can be used to extend the therapeutic window. The extent to which this might be a problem depends on how long the thiaminase needs to be present to complete its task; for example, if apoptosis is induced in a tissue as rapidly as it is induced *in vitro*, the treatment may be complete before production of antibodies became a problem. It also depends on the immunogenicity of the polypeptide used as well as the method and place of delivery. The potential toxicity and immunogenicity of various thiaminases and their derivatives, as well as their stability *in vivo*, can be monitored by regular animal testing during the development of therapeutic drugs.

In a preferred embodiment, the method includes administering an inactive analogue of the thiaminase or thiaminase derivative that shares the same epitopes,

thereby inducing immunologic tolerance to the inactive and corresponding active peptides polypeptides. Preferably, the inactive analogue is not targeted to the selected group of cells. One could create tolerance in the treated individual by flooding the system with an excess of enzymatically inactive but otherwise  
5 minimally altered thiaminase (prepared by mutagenesis, as described above in Section 3-11). Often oral provision of antigen is an efficient method of inducing tolerance (Weiner et al., 1994), but antigen given parenterally in high concentrations also induces tolerance (Dixon and Mauer, 1955).

Once multiple thiaminases are available, one could avoid the immune  
10 response by simply changing from one thiaminase to another thiaminase with different antigens during therapy.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All  
15 references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The thiamin depleting compounds and methods  
20 and accessory methods described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions  
25 and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, using other thiamin-depleting agents, targeting methods, and/or methods of administration, and other accessory methods to be used in conjunction with thiamin depletion are all within  
30 the scope of the present invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not

specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no  
5 intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and  
10 variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will  
15 recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and within the following claims.



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TABLE 1

Purification Strategy for *Naegleria* Thiaminase, with Sample Results of One  
Purification

5

<i>Fraction</i>	<i>Total protein (mg)</i>	<i>Total activity (units*)</i>	<i>Specific activity (units/mg)</i>	<i>% Yield (protein)</i>	<i>% Yield (activity)</i>	<i>Purification (fold)</i>
Crude extract	875	5.46	0.006250	100	100	—
50–70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	635	3.97	0.006257	73	73	1.001
DEAE-ion exchange	120	2.42	0.020179	14	44	3.2
HQ20 perfusion	9	1.48	0.16517	1	27	26.4
Rotofor IEF	0.225	0.425	1.892	0.02	8	302.7

\*One unit of enzyme is the amount needed to form 1  $\mu$ mol of product in 1 min at 25°C.

TABLE 2

Expressed *Naegleria* thiaminase induces apoptosis,  
and its apoptosis-inducing ability depends on thiaminase activity

5

<i>Expression clone<sup>a</sup></i>	<i>Enzyme activity assayed in</i>		<i>C6 cell death assay</i>
	<i>agar<sup>b</sup></i>	<i>cuvette<sup>c</sup></i>	<i>latent period (days)<sup>d</sup></i>
pNB1+ (wild type)	white halo	0.415 mU	4
10 pNB1-S	no halo	0.074 mU	no apoptosis

<sup>a</sup>See Example 3, Sections 3-10 and 3-11.

<sup>b</sup>See Example 1-2.

15 <sup>c</sup>See Example 1-1. Assays were performed in 1 ml volume using 10  $\mu$ l of each sample and the change in absorbance at 252 nm was measured. Absorbance was measured 10 min later and mU reported here is an average of 4 readings of each sample, which represents an increase of thiaminase activity after 24 hr of expression in *E. coli* BLR(DE3)pLysS at 37°C.

20 <sup>d</sup>See Example 2. C6 rat glioma cells were plated at 1 x 10<sup>5</sup> cells/ml in medium 199 containing 10% fetal bovine serum and 50  $\mu$ g/100 ml gentamicin and a 10<sup>-5</sup> dilution of cell extracts prepared from either of the indicated expression clones was added at the time of plating (day 0). The cultures were observed every 12 hours and the time of apoptosis noted.

25 All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

30 One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned,

as well as those inherent therein. The specific methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, those skilled in the art will recognize that the invention may suitably be practiced using a variety of different expression vectors and sequencing methods within the general descriptions provided.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is not intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group. For example, if there are alternatives A, B, and C, all of the following possibilities are included: A separately, B separately, C separately, A and B, A and C, B and C, and A and B and C.

Thus, additional embodiments are within the scope of the invention and within the following claims.

### Claims

What we claim is:

- 5           1.       A method of inducing apoptosis of a selected group of vertebrate cells *in vivo*, comprising administering to a vertebrate comprising said cells a thiaminase or derivative or a nucleic acid molecule encoding a thiaminase or derivative, thereby reducing the level of thiamin in said cells.
- 10           9.       The use of a thiaminase or derivative or nucleic acid encoding a thiaminase or derivative in the preparation of a medicament for the treatment of a disease or condition in a mammal wherein the reduction or elimination of a selected group of cells of said organism produces a therapeutic effect, and
- 15                wherein said thiaminase or derivative is able to induce apoptosis by reduction of the level of thiamin.
- 20           3.       A method for delivering a nucleic acid sequence encoding a thiaminase or derivative to vertebrate cells *in vivo*, comprising the step of contacting said cells with a vector comprising said nucleic acid sequence.
- 25           4.       A pharmaceutical composition comprising at least one thiaminase or derivative and a pharmaceutically acceptable carrier or excipient.
5.       The pharmaceutical composition of claim 4, further comprising a delivery-targeting component.
- 30           6.       The composition of claim 4, wherein said composition is sterile.

6. A method of killing a selected group of vertebrate cells *in vivo*, comprising the steps of:

a) contacting said cells with a thiaminase or thiaminase derivative, thereby reducing the level of thiamin in said cells; and

5 b) administering to a vertebrate animal having said selected group of cells an accessory treatment which enhances the effectiveness of the thiamin reduction.

10 7. A purified, enriched, or isolated nucleic acid sequence encoding a thiaminase or derivative, wherein said thiaminase agent is different from *Bacillus thiaminolyticus* thiaminase I.

15 8. The nucleic acid sequence of claim 7, wherein said thiamin-depleting agent is a *Naegleria gruberi* thiaminase or derivative, or a homolog thereof.

20 9. A eukaryotic expression vector comprising a recombinant nucleic acid sequence of claim 8.

25 10. A vector comprising a recombinant nucleic acid sequence of claim 8, wherein said thiaminase or derivative is different from a thiaminase from *Bacillus thiaminolyticus*.

11. A eukaryotic cell transformed with a eukaryotic expression vector comprising a nucleic acid sequence encoding a thiaminase or derivative.

30 12. The eukaryotic cell of claim 11, wherein said cell is *in vivo* in a vertebrate organism.



13. A composition for delivery of a nucleic acid sequence encoding a thiaminase or derivative to vertebrate cells *in vivo*, comprising:

- 5       a) a nucleic acid sequence encoding said thiaminase or derivative; and  
      b) a component associated with said nucleic acid sequence which enhances said delivery of said nucleic acid.

10       14. A method of killing a selected group of vertebrate cells *in vivo*, comprising the steps of:

- a) reducing the level of thiamin in said cells by contacting said cells with a thiaminase or derivative; and  
      b) administering to a vertebrate animal having said selected group of cells an accessory treatment which enhances the effectiveness of the thiamin  
15       reduction.

15       15. An isolated, purified, or enriched thiaminase or derivative, wherein said thiaminase is not a *Bacillus thiaminolyticus* thiaminase.

20       16. The thiaminase or derivative of claim 15, wherein said thiaminase or derivative is a homolog of a *Naegleria gruberi* thiaminase or derivative.

## Abstract

5

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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Figure 1. The *Naegleria* agent causes the rapid disappearance of thiamin from the growth medium of rat glioma C6 cells.

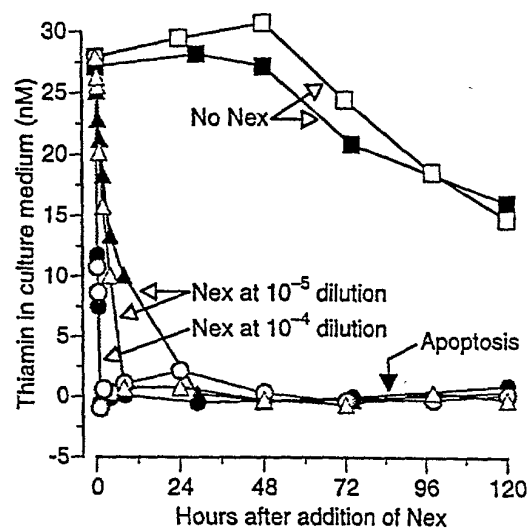


Figure 2. The addition of thiamin can reverse progress of rat glioma C6 cells toward apoptosis induced by the *Naegleria* agent. Thiamin added at 12 hour intervals reversed progress until death of the cells had already occurred on day 4.5.

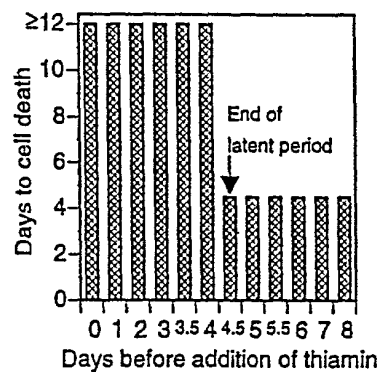


Figure 3. An SDS-polyacrylamide gel stained for thiaminase and for protein. The *Naegleria* agent was purified through preparative isoelectric focusing (see Example 3).

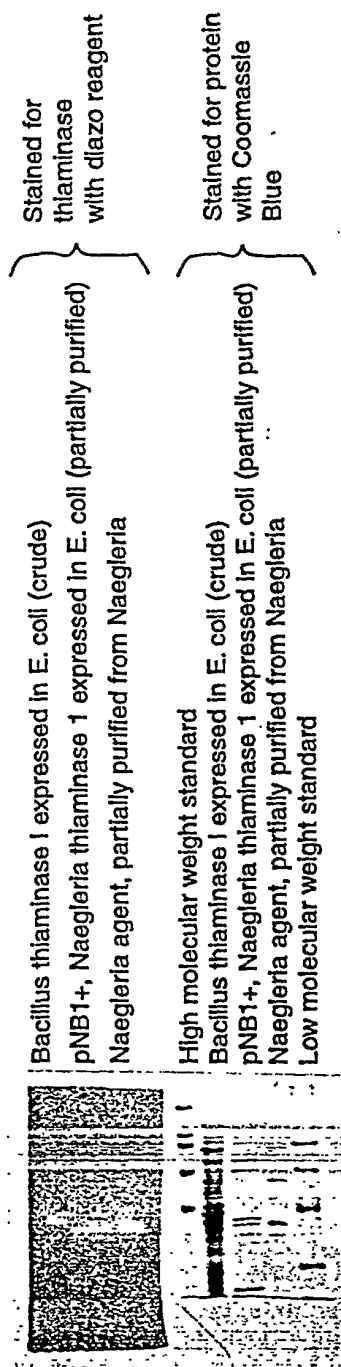


Figure 4. The complete DNA sequence of the coding region of *Naegleria* gene TTK (including the TAA stop codon). The segment of the gene that encodes thiaminase I is underlined.

ATGTCCACTCAACCAAGACACTCACTGTTGGTCTCTTCCCATATCTTCTTCTTGGAAATGAAAATGGCAACGAAGTTAA  
ATTGATCAATTTGATCAAGGATGTTTTGCCAACTCAGGTTTCCGGATATAATATCGAATATACCGAATTTGATTGTTACA  
GTGATGCTAGTCTTCAAAGTCTTCCAGATGTTTTCTCAACTGATAGCATTTCCTTCCATATCTTGTTCCTTGGGTGGT  
GTCAAGAGTTTGGATGAATCATTTGGTTCGTGGTGTACTGGTGATTGTCATAGTTTTGTTTCCTCAAGTGCCTCTGTCAA  
TGGTTCGGTTTTATGGTTTTCCACAACTACTTGTGCTCAAACTTTTATTGTCCTCACCAAATGGTACTCAACAAGCATCTT  
CCCTTTTAGAATTGGCTCAAAAAGGTTGGTTATGAACAAATGTTTATCCAGATGTTGCCTCTTCTAGTTCTTTCACAGTT  
TTCCGATTGTATCAACAATTACTCCAATCATCATCATCAGCTGCAGTTGATATCAAGGCCCTCTGATCTTCCACAATCTGG  
TGACCAAGTCAACAAGGATATCACTCAAAAATATAGAACCATTTTGGATTCAACAGTTGTTGCCTCTCAAAGAGAATATA  
TAACTCTGTAAAGCAAGGTAAACCAATTTCAAACCTACTATGTCGGATATAGTGAAAGTATGTGTGAAATTAAGGATATC  
ATCAGAGATCAACAATACAATGTTCAACTCATTTGGTACCTCTGATAAGCCATACGTTTATACTGATGTTTTGGCTTTGAA  
TTCCAAATTTGTGTGATGAAAAGCAAAAGGTTGCTGTTGAAGTTATCAAGAATTTATTGACTAATACTTTAGTTTTGGACT  
TGTTGGGTCTCGGATTAACCTCTCCAGCCAACAAGAATGGTATTGCTCATTGGCTAAATCATCAAACTTTTATGCTCAA  
TTGAGCCAACAATTCGATGCCAAGGAAAGTGAAGTTAGAGTTTGGAGATGTGTTGACTTTGCTAACAAGGAAGTTAAGAA  
TTGTGCTGGTGTCTTGAGACCATTCTTCAACATATTGCTGTTGCTACTTTGCGTTGTTGACTGCTGACACTGTCGAAA  
AGGCTAAGAGTGGTCACCCTGGTATGCCAATTGGTATGTCACCAATTGCCTATGTTTTGTGGAAGTTCTTCTTCAAATCA  
TCTAAGGATGATGTCAATTGGTTGAACAGAGATAGATTTGTTTTGAGTAATGGTCACGGTTGTACATTGCTTTATGCCAT  
GTTGCACCTCACTGATTGTAACCTTGAGTTTGGATGATCTCAAGAATTTCAGAAGTTTGCATTCCAAGACTCCTGGTCACC  
CAGAATATGGTCACACTGAAGGTGTGATGCTACTACTGGTCCATTGGGTCAAGGTGTTGTAATGCTATTGGTATGGCT  
CTCTCTGAAGCTCACTTGGCTGCTCGTTTCAATAAGGATGGACAAAATATCTTGTATCACCACACCTATGTTTTCTTGG  
TGATGGTTGTTTTGATGGAACGTGTTGCTATGGAAGGTCTCTCATTGCTGGTCACCAAAAGTTGAACAAGTTGATTGTTT  
TCTATGATGACAATAGTATTACTATTGATGGTAAGACTGAATTGACCTTTACTCAAAATACTCCAGAAGTCATGAGAGGT  
TTTGGATGGCAGCTAATTGTTGTCGACAAGGCTGATAATGACTTGGTTGGTATTAAAGGAAGCTATTTTGAAGCTCACAC  
TGTTACTGACAAGCCAATCATGATCGTTTGTAAAGACTACAATTGGTTATTCTCCTCAAAGGTTCAAGGTACTGCTAAGGTTT  
ACGGTTCTCCATTGGGTGCTGATGGATTGAAGAATTTGAAGGAACTTGTGGTTTCACTGGTAATGATTTCTTCCATGTT  
CCAGAAATTTGTCAGAAAGGACTTTGCTACTGTCAATTAATAGAAATAGTGAAGCTCTCTCAATGGAAGCAAGTTAAATC  
TGCCTATGATACCACTCATGCTACTGAATCCCAACTCCTCCAAAGAATGATTAATCAGGAATTGGAAGGTGATGTTATGG  
AAAAGTTGCCAAAATACCTCGAACAAAAGAAGATTGCTACCAGATCTACATCTCAACAAGTTTTGAATGCCATCTATCCA  
CTCATTCCTTCTCTCGTTGGTGGTTTCAAGCTGACTTGACTCCATCCAACCTGACTGATGTAAGTGGATGTCAAGATTTCCA  
ACCAAACAATAGAGTTGGTAGATATATCAGATTTGGTGTCCGTGAACATGCCATGGTTGCTATTGCCAATGGTATTTCTCT  
TTGTCTGGTCTTCCAAATATTTATGTTTTCACTCATGACAGTATTGGTCTTGGTCAAGATGGTCCAACCTCACCACCTGT  
TGAAGTTTTTACCAATGTTGATAGCCATTCCAAATCACATTGTTTTTCAAGCTGCTGATGGTAGAGAAACCAGTGGTGCTT  
ATTTGTGGGCTGTTCAATCAAAGAAGACTCCATCCTCAAGTATTCTTCTCGTCAAGATTTGCCACAATTGACTGGTACT  
GATATTTCAAAGGTTGCTTTGGGTGCCTATGTTATCCAAGGTGATGCTACTCTGATGTTGTCTTGTGGTACTGGTTC  
TGAAGTTTCCCTCATGGTTGAAGCTGCTGAAAAGTTGAAGGCTAACCTTAAGGTTAACGTTGTTTCCATGCCAAGTTGGG  
AATTGTTTTGTTGCTCAATCAGAAGAATACAGGAAGACTGTCTTCCAGATGGTATTCCAGTTGTCAGTGCCGAAGCTTCA  
TCAACCTTTGGTTGGACAAGCTTTGCTCACTATGCTGTTGGTATGACTACTTTCGGGTGCTAGTGCTGCTGCTGAAGAAGT  
TTACAAACTCCTCAAGATTACCTCAGACAATGTTGCTGAAAAGGCCACCAATTTGGTTACCAAGTATGGTAAGCAAGCTC  
CAAGACTCAGCTTGTCTCTTGTGGTGAAGAAGCTCTAA

006260.005260

Figure 5. The amino acid sequence encoded by the entire *Naegleria* TTK gene

MSTQPKTLTVGLFPYLP SWNENGNEVKLINLIKDVLP TQVSGYNIEYTEFDCYS DASLQSLPDVFSTDSIFLPYLVSLGG  
VKSLDESLVRGVTGDLHSFVSSSASVNGSVYGFPQYLCSN FLLSSPNGTQQASSLLELAQKVGYEQIVYPDVASSSSFTV  
FGLYQQLLQSSSSAAVDIKASDLPQSGDQVNKDITQKYRTILDSTVVASQREYINSVKQGKPI SNYYVGYSESMCEIKDI  
IRDQQYNVQLIGTSDKPYVYTDVLALNSNLCDEKQKVAVEVIKNLLTNTLVLDLLGLGLTLPANKNGIAHLAKSSNFY AQ  
LSQQFDAKESEVRVLRACVDFANKEVKNCAGVLRPFLQHI AVATLRCLTADTVEKAKSGHPGMPIGMSPIAYVLWKFFFS  
SKDDVNWLNRDRFVLSNGHGCTLLYAMLHLTDCNLSLDDLKNFRSLHSKTPGHPEYGHTEGVDATTGPLGQGV CNAIGMA  
LSEAHLAARFNKDGQNI FDHHTYVFLGDGCLMERVAMEGLSFAGHQKLNKLIVFYDDNSITIDGKTELFTQTNP EVMRG  
FGWHVIVVDKADNDLVGIKEAILEAHTVTDKPIMIVCKTTIGYSSKVQGTAKVHGSPLGADGLKNLKETCGFTGNDFFHV  
PEIVRKDFATVINRNSEKLSQWKQVKSAYDTTHATESQLLQRM INHELEGDMEKLPKYLEQKKIATRSTSQQVLN AIYP  
LIPSLVGG SADLTPSNLT DVTG CQDFQPNNRVGRYIRFGVREHAMVAIANGILYHGVLRTYVGTFLNFASYALGAIRLSA  
LSGLPNIYVFTHDSIGLGQDGPTHQPVEVLPMLIAIPNHIVFRPADGRETS GAYLWAVQSKKTPSSMILSRQDLPQLTGT  
DISKVALGAYVIQGDATPDVVLVGTGSEVSLMVEAAEKLKANLKVNVVSMPSWELFVRQSEYRKTVFPDGI PVVSAEAS  
STFGWTSFAHYAVGMTTFGASAAAEVYKLLKITS DNVAEKATKLVTKY GKQAPRLSLSLVGEEL

005260-605250

[illegible]

ATGTCCACTCAACCAAAGACTCACTGTTGGTCTCTTCCCATATCTTCTCTTGGAAATGAAATGGCAACGAAGTTAA  
ATTGATCAATTTGATCAAGGATGTTTTGCCAACTCAGGTTTCCGGATATAATATCGAATATACCGAATTTGATTGTTACA  
GTGATGCTAGTCTTCAAAGTCTTCCAGATGTTTTCTCAACTGATAGCATTTTCTTCCATATCTTGTTTCTTTGGGTGGT  
GTCAAGAGTTTGGATGAATCATTGGTTCGTGGTGTTACTGGTGATTGTCATAGTTTTGTTTCCCTCAAGTGCCTCTGTCAA  
TGGTTCGGTTTATGTTTTCCACAATACTTGTGCTCAAACTTTTATTGTCTCACCAAATGGTACTCAACAAGCATCTT  
CCCTTTTAGAATTGGCTCAAAGGTTGGTTATGAACAAATTGTTTATCCAGATGTTGCCTCTTCTAGTCTTTACAGTT  
TTCGGATTGTATCAACAATTACTCCAATCATCATCAGCTGCAGTTGATATCAAGGCCTCTGATCTCCACAATCTGG  
TGACCAAGTCAACAAGGATATCACTCAAAAATATAGAACCATTTTGGATTCAACAGTTGTTGCCTCTCAAAGAGAATATA  
TTAACTCTGTAAAGCAAGGTAACCAATTTCAAACACTATGTGCGATATAGTGAAAGTATGTGTGAAATTAAGGATATC  
ATCAGAGATCAACAATACAATGTTCAACTCATTGGTACCTCTGATAAGCCATACGTTTATACTGATGTTTTGGCTTTGAA  
TTCCAATTTGTGTGATGAAAGCAAAAGGTTGCTGTTGAAGTTATCAAGAATTTATTGACTAATACTTTAGTTTTGGACT  
TGTTGGGTCTCGGATTAACTCTCCAGCCAAACAAGATGGTATTGCTCATTGGCTAAATCATCAAACTTTTATGCTCAA  
TTGAGCCAACAATTCGATGCCAAGGAAAGTGAAGTTAGAGTTTGGAGATGTGTTGACTTTGCTAACAAAGGAAGTTAAGAA  
TTGTGCTGGTGTCTTGAGACCATTCTT



Figure 7. The encoded amino acid sequence of the expressed *Naegleria* thiaminase I gene, along with its codons, and with residues numbered (nucleotide/amino acid).

1/1 31/11  
ATG TCC ACT CAA CCA AAG ACA CTC ACT GTT GGT CTC TTC CCA TAT CTT CCT TCT TGG AAT  
Met ser thr gln pro lys thr leu thr val gly leu phe pro tyr leu pro ser trp asn

61/21 91/31  
GAA AAT GGC AAC GAA GTT AAA TTG ATC AAT TTG ATC AAG GAT GTT TTG CCA ACT CAG GTT  
glu asn gly asn glu val lys leu ile asn leu ile lys asp val leu pro thr gln val

121/41 151/51  
TCC GGA TAT AAT ATC GAA TAT ACC GAA TTT GAT TGT TAC AGT GAT GCT AGT CTT CAA AGT  
ser gly tyr asn ile glu tyr thr glu phe asp cys tyr ser asp ala ser leu gln ser

181/61 211/71  
CTT CCA GAT GTT TTC TCA ACT GAT AGC ATT TTC CTT CCA TAT CTT GTT TCT TTG GGT GGT  
leu pro asp val phe ser thr asp ser ile phe leu pro tyr leu val ser leu gly gly

241/81 271/91  
GTC AAG AGT TTG GAT GAA TCA TTG GTT CGT GGT GTT ACT GGT GAT TTG CAT AGT TTT GTT  
val lys ser leu asp glu ser leu val arg gly val thr gly asp leu his ser phe val

301/101 331/111  
TCC TCA AGT GCC TCT GTC AAT GGT TCC GTT TAT GGT TTC CCA CAA TAC TTG TGC TCA AAC  
ser ser ser ala ser val asn gly ser val tyr gly phe pro gln tyr leu cys ser asn

361/121 391/131  
TTT TTA TTG TCC TCA CCA AAT GGT ACT CAA CAA GCA TCT TCC CTT TTA GAA TTG GCT CAA  
phe leu leu ser ser pro asn gly thr gln gln ala ser ser leu leu glu leu ala gln

421/141 451/151  
AAG GTT GGT TAT GAA CAA ATT GTT TAT CCA GAT GTT GCC TCT TCT AGT TCT TTC ACA GTT  
lys val gly tyr glu gln ile val tyr pro asp val ala ser ser ser ser phe thr val

481/161 511/171  
TTC GGA TTG TAT CAA CAA TTA CTC CAA TCA TCA TCA TCA GCT GCA GTT GAT ATC AAG GCC  
phe gly leu tyr gln gln leu leu gln ser ser ser ser ala ala val asp ile lys ala

541/181 571/191  
TCT GAT CTT CCA CAA TCT GGT GAC CAA GTC AAC AAG GAT ATC ACT CAA AAA TAT AGA ACC  
ser asp leu pro gln ser gly asp gln val asn lys asp ile thr gln lys tyr arg thr

601/201 631/211  
ATT TTG GAT TCA ACA GTT GTT GCC TCT CAA AGA GAA TAT ATT AAC TCT GTA AAG CAA GGT  
ile leu asp ser thr val val ala ser gln arg glu tyr ile asn ser val lys gln gly

661/221 691/231  
AAA CCA ATT TCA AAC TAC TAT GTC GGA TAT AGT GAA AGT ATG TGT GAA ATT AAG GAT ATC  
lys pro ile ser asn tyr tyr val gly tyr ser glu ser met cys glu ile lys asp ile

721/241 751/251  
ATC AGA GAT CAA CAA TAC AAT GTT CAA CTC ATT GGT ACC TCT GAT AAG CCA TAC GTT TAT  
ile arg asp gln gln tyr asn val gln leu ile gly thr ser asp lys pro tyr val tyr

781/261 811/271  
ACT GAT GTT TTG GCT TTG AAT TCC AAT TTG TGT GAT GAA AAG CAA AAG GTT GCT GTT GAA  
thr asp val leu ala leu asn ser asn leu cys asp glu lys gln lys val ala val glu

841/281 871/291  
GTT ATC AAG AAT TTA TTG ACT AAT ACT TTA GTT TTG GAC TTG TTG GGT CTC GGA TTA ACT  
val ile lys asn leu leu thr asn thr leu val leu asp leu leu gly leu gly leu thr l

901/301 931/311  
CTC CCA GCC AAC AAG AAT GGT ATT GCT CAT TTG GCT AAA TCA TCA AAC TTT TAT GCT CAA  
leu pro ala asn lys asn gly ile ala his leu ala lys ser ser asn phe tyr ala gln

961/321 991/331  
TTG AGC CAA CAA TTC GAT GCC AAG GAA AGT GAA GTT AGA GTT TTG AGA TGT GTT GAC TTT  
leu ser gln gln phe asp ala lys glu ser glu val arg val leu arg cys val asp phe

1021/341 1051/351  
GCT AAC AAG GAA GTT AAG AAT TGT GCT GGT GTC TTG AGA CCA TTC CTT  
ala asn lys glu val lys asn cys ala gly val leu arg pro phe leu

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Figure 8. Comparison of the amino acid sequence of *Naegleria* thiaminase 1 (N40KAT) aligned with the sequences of *Bacillus* thiaminase (BSTP) and segments of the transketolases of six organisms. The numbering system indicated here is for the yeast transketolase.

Abbrev.	Genbank	Enzyme and Organism
ScTKT1ct -	P23254	Transketolase of <i>Saccharomyces cerevisiae</i>
CpTKT7ct -	Q42677	Transketolase of <i>Craterostigma plantagineum</i>
EcTKT2ct -	P33570	Transketolase of <i>Escherichia coli</i>
BsTKTct -	P45694	Transketolase of <i>Bacillus subtilis</i>
MgTKT -	P47312	Transketolase of <i>Mycoplasma genitalium</i>
MjPTK1 -	Q58092	Transketolase of <i>Methanococcus jannaschii</i>
BSTP -	P45741	Thiaminase I precursor from <i>Bacillus thiaminolyticus</i>
N40KAT -	----->	Thiaminase I of <i>Naegleria gruberi</i> , aa 1-356

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ScTKT1ct      ADDVK---QLKSKFGFNPDKSFVVPQEVYD-HYQKTILKPGVEANNKWNKLFSEYQKKFP      56
CpTKT7ct      PKEAE---ATRKNLGW-PYEPFHVDPDDVKK-HWSRHIAE-GAALESANNAKFAEFQKKFP
EcTKT2ct      EEEVA---LARQKLGW-HHPPFEIPKEIY--HAWDAREK-GEKAQQSWNEKFAAYKKAHP
BsTKTct       KEESK---LTKEAYAWTYEEDFYVPSEVYE-HFAVAVKESGEKKEQEWNAQFAKYKEVYP
MgTKT         EVDFQ---LFEKRTNT-NFNFFNYPDSIYH-WFKQTVIERQKQIKEDYNNLLISLKD-K-P
MjPTK1
BSTP
N40KAT        MSKVKGFIYKPLMVMLALLLVVSPAGAGAAHSDASSDITLKVAIYPYVPDPAFQAQAVL
MSTQP-----KTLTVGLFPYL-----PS-----WNENGNEVKLINLIKDVLP-----

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ScTKT1ct      ELGAELARRLSGQLPANWESKLPTYTAKDSA----VATRKLSETVLEDVYNQLPELIGGS      112
CpTKT7ct      EEAADLKSIIITGELPTNWESIFPTYTPENPG----LPTRTLSHQIILNGLGDVLPGLLGGS
EcTKT2ct      QLAEFTRRMSGGLPKDWKKTQKYINELQANPAKIATRKASQNTLNAYGPMPLPELLGGS
BsTKTct       ELAEQLELAIKGELPKDWQEVVPVYE-KGSS----LASRASSGEVLNGLAKKIPFFVGGS
MgTKT         -LFFKFTNWIDSDFQALYLNQLDEKKVAKKD----SATRNYLKDFLNQINNPNNSNLYCLN
MjPTK1        -----MVKLSGVYKG-----MRKGYGETLIELGKKYENLVVLD
BSTP         DQWQRQEPGVKLEF-TDWDSSYADPPDDLVD----FVLDSIFLSHFVDAGYLLP-FGSQD
N40KAT        -----QVSGYNIEY-TEFDCYSASLQSLPD---VFSTDSIFLPYLVSLGGVKSLLDESIV

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ScTKT1ct      ADLTPSNLTRWKEALDFQPPSSSGSGNYSGRYIRYGIREHAMGA---IMNGISAFGANYPYGG      172
CpTKT7ct      ADLTLNMAFLKNSGDFQKKSPGE-----RNVKFGAREHAMGS---ICNGLALHSPGLLPYCA
EcTKT2ct      ADLAPSNLTIWKGVSVLKEDPAGN-----YIHYGVRFGMTA---IANGIAHHG-GFVPTYA
BsTKTct       ADLAGSNKTTIKNAGDFTAVDYSG-----KNFWFGVREFAMGA---ALNGMALHG-GLRVFGG
MgTKT         ADVSR--CFIKIGDDNLHENPCS-----RNIQIGIREFAMAT---IMNGMALHG-GIKVMGG
MjPTK1        ADLSGS-----TQTAMFAKEFPE-----RFFNAGVAEQNMIG---MAAGLATTG--KIVFAS
BSTP         IDQAEDVLPFALQGAQRNGEVYGLP-----QILCTNLLFYRKGDCLKIGQVDNIYELYKKIG
N40KAT        RGVGTGDLHSFVSSASVNGSVYGF-----QYLCNFFLLSS-----PNGTQQAS-SLELAQ

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Catalytic Cys

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ScTKT1ct      TFLNFVS-YAAGAVR-LSALSGHFVIWVATHDSIGV-GE----DG-PTHQPIET--LAHF      222
CpTKT7ct      TYFVFTD-YMRAAMR-ISALSARVLYIMTHDSIGL-GE----DG-PTHQPVVEH--LASF
EcTKT2ct      TFLMFVE-YARNAAR-MAALMKARQIMVYTHDSIGL-GE----DG-PTHQAVEQ--LASL
BsTKTct       TFFVFS-D-YLRPAIR-LAALMGLPVTVYVTHDSIAV-GE----DG-PTHEPVEQ--LASL
MgTKT         TFLAFAD-YSKPAIR-LGALMNLFPVYVYTHDSYQV-GG----DG-PTHQPYDQ--LPML
MjPTK1        SFSMFASGRAWEIIRNLVAYPKLVKIVATHAGITV-GE----DG-ASHQMCED--IAIM
BSTP         TSHSEQIPPPQNKGLLINMAGGTTKASMYLEALIDVTGQYTEYDLLPPLDPLNDKVIRGL
N40KAT        KVGYEQIVYPDVASSSSFTVFGLYQQLLQSSSSAAV-----DIKASDLPQSGD-QVNK

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Peptide A -> ASDLPQSGD-QVNK

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Figure 8, continued.

ScTKT1ct	RSLPNIQVWRPADGN-EVSAAYKNSLESKHTPSIIALSRLQNPQLEGS---SIESASKGG	278
CpTKT7ct	RAMPNILLRTPADGN-ETAGAYRAAVQNGERPSILVLRQKLPQLPGT---SIEGVSKGG	
EcTKT2ct	RLTPNFSTWRPCDQV-EAAVGVKLAVERHNGPTALILSRQNLQVVERTPD-QVKEIARGG	
BsTKTct	RAMPNLSLIRPADGN-ETAAAWKLAVQSTDHPTALVLRQNLPTIDQTSEEALAGVEKGA	
MgTKT	RAIENVCVFRPCDEK-ETCAGFNYGLLSQDQTTVLVLRQPLKSIDNTD--SLKTL-KGG	
MjPTK1	RAIPNMVVIAPTDYY-HTKNVIRTIAEYKG-PVYVRMPRRDTEIIYENEEATFEIGKKG	
BsTP	RLLINMAGEKPSQYVPEDGDAYVRASWFAQ-GSGRAFIGYSESMMRMG---DYAEQVRFK	
N40KAT	DITQKYRTILDSTVV-ASQREYINSVKQKG-PISNYVGYSESMCEIK---DIIRDQQYN	

Peptide B -> TILDSTVV-ASQR

ScTKT1ct	YVLQDVAN-----PDIILVATGSEVSLSVEAAKTLAAKNIKARVVSLEPDDFTFDKQPLE-	332
CpTKT7ct	YVIDNSRGGNSKPDVILIGTGSELEIAARAGDELKKEGKKVRVSVLVCWELFAEQSEK-	
EcTKT2ct	YVLKDSGG---KPDIIILVATGSEMEITLQAAEKLAGEGRNVRVSLPSTDFDAQDEE-	
BsTKTct	YVVSXSKN--E-TPDALLIASGSEVGLAIEAQELAKENIDVSVVSMPSMDRFKQSD-	
MgTKT	YILLDRKQ-----PDLIIAASGSEVQLAIEFEKVLTKQNVKVRILSVPNITLLLKQDEK-	
MjPTK1	ILVDG-----EDLTIIATGEEVPEALRAGEILKENGISAEIVEMATIKPIDEEIHK	
BsTP	PISSSAG-----QDIPLFYSDVSVNSKTAHPELAKKLANVMSADTVEQALRPQADGQ	
N40KAT	VQLIGTS-----DKPYVYTDVLAALNSN---LCDEKQKVAVEVIKLLTNTLVLDLLG-	

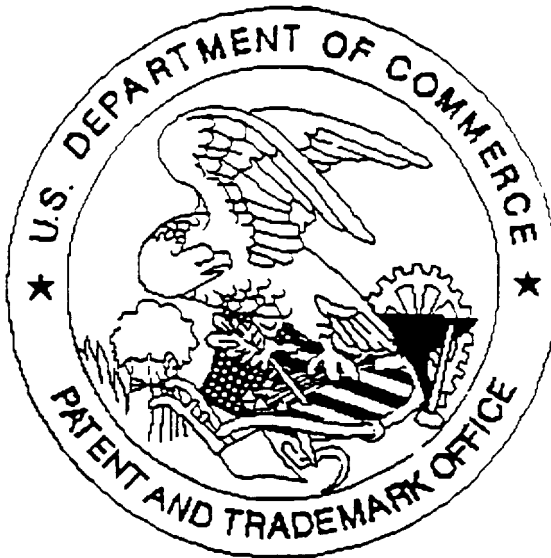
ScTKT1ct	YRLSVLPDNPVI-MSVEVLATTTCWGKYAH-----QSFGIDRFGASGKAPEVFKFFGFTP	385
CpTKT7ct	YRETVLPSTVARTARVSEAGSTFGWERFIGP-KG--KAVGIDRFGASAPAERLFKEFGITV	
EcTKT2ct	YRESVLPSNVAARVAVEAGIADYWKYVGL-KG--AIVGMTGYGESAPADKLFPPFGFTA	
BsTKTct	YKNEVLPAADVKKRLAIEMGSSFGWGKYTGL-EG--DVLGIDRFGASAPGETIINEYGFSV	
MgTKT	YLKSLFDANSSL-ITIEASSSYEWFCFKKY-VKNHAHLGAFSFGESDDGDKVYQQKGFNL	
MjPTK1	SKDFVVTVEDHSIIIGGLGGAVAIEVIAENGLNKKLLRIGINVDFGRSGKADELLKYGLDG	
BsTP	YPQYLLPARHQV-YEALMQDYPIYSELAQIVNK--PSNRVFRLGPEVRT-WLKDAKQVLP	
N40KAT	-LGLTLTPANKNG-IAHLAKSSNFYAQLSQ-----QFDAKESEVRVLRVDFANKEV	

Peptide C -> SSNFYAQLSQ-----QFDAK

ScTKT1ct	EGVAERAQKTIAFYKGDKLISPLKKA	412
CpTKT7ct	EAVVA-AAKEIC-----	
EcTKT2ct	ENIVAKAHKVLGVKGA-----	
BsTKTct	PNVVNRVKALINK-----	
MgTKT	ERLMKIFTSLRN-----	
MjPTK1	ESIAKRIMEEMKKE-----	
BsTP	EALGLTDVSSLAS-----	
N40KAT	KNCAGVLRPFL-----	

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